ON THE MECHANISM OF ACTION OF INTERSTITIAL CELL-STIMULATING HORMONE

by

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PREFACE

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ABBREVIATIONS

The abbreviations used are those recommended in Style Mannual For Biological Journals. American Institute of Biological Sciences. Washington. 1960. In addition the following abbreviations have been used.

ACTH	Adrenocorticotrophic Hormone
ADH	Antidiuretic Hormone (Vasopressin)
Androstenedione	Δ^4-androstene-3,17-dione
ATP	Adenosine triphosphate
CM Cellulose	Carboxymethyl cellulose
CoA	Coenzyme A
CPM	Counts per minute
Cyclic AMP	Adenosine-3',5'-cyclic monophosphate
DEAE Cellulose	Diethylaminoethyl cellulose
DHEA	Dehydroepiandrosterone
DPM	Disintegrations per minute
DPN	Diphosphopyridine nucleotide
FSH	Follicle-stimulating hormone
Glucose-6-PO_4	Glucose-6-phosphate
HCG	Human chorionic gonadotrophin
17-α-Hydroxypregnenolone	Pregn-5-ene-3β,17α-diol-20-one
ICSH	Interstitial cell-stimulating hormone
MSH	Melanocyte-stimulating hormone

Pregnenolone	Pregn-5-en-3β-ol-20-one
TCA	Trichloracetic acid
TPN	Triphosphopyridine nucleotide

SPELLING

1. Gonadotrophin will be spelled with an "h".

2. Hypophysial will be spelled with an "i", which is the form agreed upon by Rioch, D. McK. et al. in The Hypothalamus and Control Levels of Autonomic Function. The Williams and Wilkins Company. Baltimore 1940. Page 3.

NUMBERING OF EXPERIMENTS

The numbers given to experiments in the text refer to the numbering system used in the Journal of Experiments volumes I-IV kept in the laboratory of the Department of Biochemistry, University of Utah, Salt Lake City.

CHAPTER I

BACKGROUND AND PROBLEM

Introduction

The experiments to be described deal with the mechanism of action of gonadotrophic hormones upon the biosynthetic activity of testicular tissue. Such experiments have become possible as the result of three major developments in this field. Firstly, the purification of gonadotrophic hormones has been accomplished on a relatively large scale, with the result that generous quantities of highly purified preparations have become available. Secondly, the biosynthetic pathways leading to the production of steroid compounds have been intensively studied and thirdly, the mechanism of action of corticotrophin upon the adrenal cortex has been the subject of numerous important investigations. The present chapter attempts to correlate current concepts of these diverse developments and replaces a formal historical treatment of the remote events to which our present understanding of the subject can be traced. The literature is reviewed under the following headings:

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I. GONADOTROPHIC HORMONES

A. The Nature and Number of Pituitary Gonadotrophic Hormones.

The results of hypophysectomy in both adult and immature animals leave no doubt that normal gonadal function is, in part at least, dependent upon the activity of the adenohypophysis (1,2). Fluhman, for example, in 1927 gave a masterly account of this relationship in the case of the ovary. It has, moreover, been long realized that the gonads in turn affect the pituitary, since castration is followed by striking histological changes in the cells of the adenohypophysis (3).

Many of the early experiments were performed on female animals and progress was delayed by the discovery that extracts from or implants of pituitary tissue failed to initiate and to maintain adult ovarian function in immature ovaries. In many cases the phenomenon of luteinization without ovulation was seen - the corpora lutea containing ova (4). However, two independent reports appeared within one year announcing the discovery of a gonadotrophic substance in blood and urine respectively, of menopausal women (5,6); both substances caused growth of ovarian follicles. These findings were logically taken to indicate the existence of two gonadotrophic hormones, one causing follicular growth and the other causing the development of corpora lutea.

Accordingly, attempts were made to isolate these hormones from

pituitary tissue and this was achieved to the extent that two proteins were isolated, each showing predominantly one or other effect, but neither being entirely free of the other. Numerous workers have endeavored to enhance the purity of these substances but so far it would not be safe to say that either hormone is available in a state of absolute purity. Nevertheless, highly purified preparations are at present available and the physico-chemical properties of these are sufficiently distinct to justify the belief that, apart from prolactin, sheep, swine and human pituitary glands contain two gonadotrophins. Table I shows some of the important differences between the properties of the two sheep hormones.

Table I

Comparison of Physico-chemical Properties of Sheep FSH and ICSH (after Steelman and Segaloff (51))

Property	FSH	ICSH
Isoelectric Point (pH)	<5.0	7.3
Molecular Weight	25,000-30,000	30,000
DEAE Cellulose pH 7.0 0.01 M NaCl	Retained	Not retained
CM Cellulose pH 6.0 0.01 M NaCl	Not retained	Retained
Hydroxyapatite pH 6.8 0.04 M PO_4	Not retained	Retained
Stability to Pancreatin	Stable	Unstable
Stability in lyophilized state	Unstable	Stable

In spite of such findings and of the gross differences in the biological actions of the two hormones, some workers (7) have suggested that the pituitary produces one gonadotrophic hormone with

both follicle-stimulating and luteinizing actions and that the two preparations which appear to possess only one or other activity, are biological or chemical artefacts, appearing during extraction or purification. There is little to support such a view except that the adenohypophysis possesses highly active proteolytic enzymes (8) which could possibly attack a protein molecule during extraction and that the unequivocal identification of two distinct gonadotrophins in human urine has yet to be achieved (9). However, Squire and Li (33) have been careful to consider the first possibility and accordingly extract at a pH well below the optimum of the more prevalent proteolytic enzyme found in sheep pituitary tissue. On the other hand, the difficulty of identifying two urinary gonadotrophins may represent a purely technical problem arising from such factors as the variable and complex composition of urine or may reflect differences in the metabolism or excretion of the two hormones. Apostolakis and Loraine have recently shown that the renal clearance of human pituitary gonadotrophins is low (10), but as yet the relative clearance of the two gonadotrophins has not been studied, although it has been shown, by investigations of patients subjected to hypophysectomy, that human urinary gonadotrophins are of pituitary origin (12).

It has been clearly demonstrated that two gonadotrophic activities are displayed by the pituitary tissues of sheep (85), swine (27) and man (48). The isolation of two physico-chemically distinct and highly purified proteins from the glands of sheep, swine and man strongly supports the existence of two hormones. In what follows, it will be assumed that the adenohypophysis secretes (in addition to prolactin)

two gonadotrophins, namely follicle-stimulating hormone (FSH) and interstitial cell-stimulating hormone (ICSH) and that these two hormones are released into the blood stream, although their separate identification in plasma has yet to be achieved.

Following the first crude separation of the two gonadotrophins from pituitary tissue derived from animals of both sexes, it was shown that the same hormones were active in hypophysectomized male animals, FSH being spermatogenic while ICSH stimulated the Leydig cells (98,99). Moreover, hypophysectomy was shown to be followed by striking changes in the interstitial cells of both the ovary and the testis (14,21). In the case of the ovary, these cells became smaller and showed dense chromatin-staining material (so-called agmination of chromatin), which gave the cells a characteristic dense appearance readily detected with the low power of a microscope (4,14, 21). Selye described these changes with the name "deficiency cells" (4) and Simpson (14,21) described analogous changes in the Leydig cells. In both cases these changes were reversed by the administration of ICSH (4,14,21).

Unequivocal evidence was soon available that a single preparation of FSH was capable of stimulating both ovarian follicular development and spermatogenesis, but some objection was raised to the assumption that the hormone which caused luteinization was identical with that which stimulated interstitial cells (14). However, Fevold showed that the one hormone produced both effects (15), thus disposing of the contention of Evans et al. that ICSH could stimulate only the interstitial cells of the ovary while the luteinizing hormone could also produce luteinization (14).

B. The Nomenclature of Pituitary Gonadotrophic Hormones.

If the pituitary secretes two gonadotrophins and if these hormones are active in both sexes, the problem of nomenclature arises. Since the two hormones derived from either sex exert analogous effects upon both the testis and the ovary, it would seem desirable to use the same names for both hormones, regardless of the sex of the animals from which they are derived. It must, however, be realized that the exact structural identity of the hormones in both sexes has not been established and that the present source of both hormones is slaughter-house material containing an unspecified mixture of pituitary tissue from both sexes. Such a scheme would suggest the names follicle-stimulating hormone and interstitial cell-stimulating hormone, which, however, have certain disadvantages. In the first place, these names are awkward and in the second place, follicle stimulation is confined to the ovary, while interstitial cell stimulation refers, in the case of the ovary, to a much less conspicuous response than the dramatic phenomenon of luteinization. If the last objection is met by using the term luteinizing hormone for the hormone derived from female animals and thus adopting three names for two hormones, we unwittingly suggest an identity between the sexes in the case of follicle-stimulating hormone which is lacking in the case of interstitial cell-stimulating hormone.

Van Dyke and Coffin suggested resolving this dilemma by adopting the names thylakentrin and metakentrin (16). Kentrin, meaning "to goad or stimulate", is common to both words. Follicle-stimulating hormone would then be called thylakentrin, thylos being a bag or sac. Aristotle used the word thylos to refer to the sac in which the eggs

of the tunny are enveloped and by a happy chance the noun θγλα′Κη is the Greek word for scrotum, so that one name might serve for both sexes. Metakentrin was proposed for interstitial cell-stimulating hormone, meta meaning "between" or "among". Unfortunately, these names have never received popular approval and the hope is respectfully expressed that they may be resurrected to receive the blessing of an international congress and a second chance to reveal their worth. In the present work the names follicle-stimulating hormone and interstitial cell-stimulating hormone will be used and will appear in the abbreviated forms FSH and ICSH.

C. The Purification and Presently Available Preparations of Pituitary Gonadotrophic Hormones.

In 1931, Fevold and coworkers published an account of the first satisfactory method for the preparation of relatively pure gonadotrophins from sheep pituitary (17). This method was based upon the solubility of FSH in water following aqueous pyridine extraction of the pituitary. In 1939, Jensen and coworkers in Li's laboratory, described a different method for the isolation of the two hormones in the same species (18), while in the same year the group at the Squibb Institute for Medical Research (Greep, vanDyke and Chow) separated the two hormones from swine pituitary with the aid of proteolytic enzymes (19); this method was based upon observations of McShan and Meyer who showed that trypsin destroyed the activity of ICSH, but not that of FSH (20).

The following year brought publications from both laboratories. Li's group modified the method of 1939 (22) and reported on the

physico-chemical and biological properties of sheep ICSH (21) and on the pituitary content of ICSH (23). Meanwhile, Greep and colleagues succeeded in preparing the swine hormones without preliminary proteolytic digestion (24) and achieved three aims, namely:

(1) separation of the two gonadotrophins in one stage without serious loss,

(2) evidence of biological potency and purity of the hormones in hypophysectomized rats, and

(3) physico-chemical evidence that the hormones were nearly pure.

In a second paper the same year, Greep et al. announced that their preparation of swine ICSH was homogeneous to electrophoresis, ultracentrifugation and constant solubility (25). The minimal active dose in the hypophysectomized male rat was 6.7 µg. In the female, their ICSH caused luteinization if follicles were present.

Later in 1940, Li et al. indicated that their preparation of sheep ICSH was homogeneous to electrophoresis but differed strikingly in physico-chemical properties from the swine preparation of Greep et al. (26).

The Squibb group in 1942 summarized their experience with swine FSH and ICSH in a series of three masterly papers (27-29). They had simplified the original method of preparing ICSH by precipitation at the isoelectric point. Their findings indicated that both preparations were, according to tests of biological activity, highly purified.

In the ensuing years, protein chemists came to realize that the criteria of purity in the case of proteins had not been sufficiently rigorous (30). As existing techniques were refined and methods such

as zone electrophoresis were introduced, it was seen that absolute purity was an ideal not attained in the case of most existing protein preparations and, moreover that the purity of proteins should be defined in terms of the operations used to examine such purity. In short, it was realized that "purity" was assumed if homogeneity was observed in a number of laboratory procedures which were required to be specified in each case. Consequently, the purity of protein hormones was examined more critically. For example, in 1958 Squire and Li submitted their 1940 preparation of sheep ICSH (Li et al. 1940 (21)) to chromatography on IRC-50 resin (XE-97) and separated two fractions, one of which was purified by zone electrophoresis on starch and rechromatography on IRC-50 (31). The product was called β-ICSH.

The value of ion exchange resins for the selective adsorption of ICSH was clearly demonstrated by Leonora and coworkers in the same year (32). These workers used the anion resin XE-59 at pH 9, followed by the cation resin XE-97. The following year Squire and Li published a full account of the preparation of highly purified β-ICSH (33). The procedure involves precipitation with alcohol, ammonium sulfate and sulfosalicylate, chromatography on the resin Amberlite IRC-50, zone electrophoresis on starch at pH 4.2 and finally, by rechromatography. The material obtained was homogeneous to zone electrophoresis, boundary electrophoresis, ultracentrifugation and diffusion. The process yielded 600 mg of β-ICSH from 1 kg of pituitary and the molecular weight was calculated to be 40,000.

In 1959, Ward and colleagues confirmed the occurrence of two chromatographically distinct ICSH fractions from sheep pituitary

which they separated by means of the ion-exchange agents, carboxymethyl-cellulose and hydroxyapatite (34); both fractions were shown to possess ICSH activity in the ventral prostate test using both hypophysectomized and intact rats. Ward called the slower moving compound LH_2. The faster moving LH_1 on rechromatography was partly dissociated into LH_2 and a substance not retained by the column. The authors showed that LH_1 and LH_2 were approximately equal in potency to the Armour standard LH 227-80. Viscosity and ultracentrifugal data indicated a molecular weight of 38,000 for LH_2. A later paper indicated that LH_1 was a chromatographic artefact formed between LH_2 and an acid protein present in the crude gonadotrophic fraction before chromatography (35).

These disturbing findings indicate that experience with other proteins (30) has repeated itself with ICSH, which can only be regarded as pure by specified criteria and judgment should be reserved concerning the possible presence of small amounts of contaminants. Squire and Li, however, state that their β-ICSH contains no other pituitary hormones in concentrations greater than 0.1 per cent (33). The occurrence of two fractions of ICSH originally raised the possibility of the secretion of numerous gonadotrophins but artifactitious association with other proteins appears to be a more probable explanation for such fractions.

Isolation of multiple ICSH fractions has also been reported by Jutisz and Squire (36) and by Leonora *et al.* (32), while association of gonadotrophins with inactive proteins has been reported by Bourrillon *et al.* (37,38). It seems possible that this phenomenon is not uncommon and should be kept in mind when the purity of gonadotrophins is under discussion.

Meanwhile progress was being made with the purification of FSH. In 1940, a relatively pure preparation of sheep FSH was achieved in Li's laboratory (39), and the method was later modified using ethanol precipitation, dialysis and ammonium sulfate fractionation (40). This method gave a substance which was homogeneous to electrophoresis, to dialysis and to ultracentrifugation. In 1950, vanDyke and coworkers found that the most satisfactory method of preparing swine FSH was by salt fractionation with ammonium sulfate (41), while in 1953, Steelman and colleagues used acid acetone extraction to isolate FSH from the same species (42). Two years later Steelman and his group submitted their preparation to digestion with pancreatin at 37°C for 3 hours, using an enzyme to substrate ratio of 1:100 (43). In spite of a considerable increase in activity, the product of digestion was not homogeneous to ultracentrifugation, while electrophoresis indicated the presence of five components. In 1956, the same workers gave a full account of their method of purification and showed that the bulk of the material was composed of contaminants (44,45). However, homogeneity was achieved with the aid of DEAE cellulose to which the digested material was applied at neutral pH and low ionic strength. Elution with 0.5 M sodium chloride and 0.1 M disodium hydrogen phosphate was followed by dialysis and rechromatography. The final product was shown to have a molecular weight of 29,000 and to be 30 to 50 times as potent as the Armour standard (264-151-X); 1-2.5 µg of Steelman's material caused a significant increase in the ovarian weight of rats (45).

As in the case of ICSH, Li and colleagues in 1958 demonstrated the value of zone electrophoresis, which purified their FSH preparation

of 1949 tenfold but still left the hormone inhomogeneous to zone electrophoresis (46). Indeed, the major protein component of the preparation studied by zone electrophoresis proved to be biologically inert. In the same year, Li et al. prepared FSH from human pituitary and purified the hormone 15-fold by ammonium sulfate fractionation together with a cation exchange resin (47). In 1960, the same group published a full account of a method for the isolation of human FSH and ICSH (48). Steelman and coworkers have also published a method for the preparation of highly active human FSH (49).

Ellis made a valuable contribution to the field in 1958 by publishing a scheme for the recovery of four anterior pituitary hormones in one procedure (50). The method involves the successive use of metaphosphoric acid and ethanol precipitation, chromatography on diethylaminoethyl cellulose and zone electrophoresis. Using sheep pituitary the author reported the isolation of FSH 30-40 times as active as the Armour Standard (264-151-X). The ICSH produced was purified 50-fold by adsorption on a cation exchange resin. This method of purification has been used in the preparation of most of the gonadotrophins used in the experiments to be described.

Steelman has reviewed the procedures in current use for the separation and purification of FSH and ICSH (51) and points out that those which are successful in this and which also make provision for the recovery of other pituitary hormones as by-products, are based upon two principal methods, namely:

(1) the ethanol acetate method of Koenig and King (52), and

(2) the Steelman acid acetone method (42).

The Koenig-King method is successful in ovine and human glands (52),

the Steelman method with swine and horse pituitary (60), but not with bovine or ovine glands. The Steelman procedure is designed to follow Lyon's acid-acetone treatment for the recovery of ACTH (53) and is based upon rapid neutralization and alkaline extraction of the residue (Step I porcine FSH) following this treatment. The precipitate which appears on cooling the alkaline ethanol extract is called Step II porcine FSH and from this the Armour FSH standard (264-151X) was prepared by the use of repeated ammonium sulfate precipitation (42).

The surprising impurity of FSH preparations was revealed when Steelman showed that the preparation of Li et al. (1949) (40), from sheep, that of vanDyke et al. (1950) (41) from swine and the Armour standard from sheep were all of approximately equal potency, while certain of his own porcine preparations were twice as potent as these extracts and yet were clearly heterogeneous (51). Further examination of FSH preparations which had been regarded as relatively pure, confirmed these doubts, as follows:

1. Steelman subjected his own swine preparation to fractional, electrical transport studies only to find that it was far from homogeneous (44).

2. Ward and Steelman purified specimens of swine FSH by chromatography on carboxymethyl cellulose and on hydroxyapatite. These methods also revealed that the preparations tested were not homogeneous (34,54).

3. Ellis and Steelman showed that chromatography on DEAE cellulose also revealed contaminants in highly purified FSH (65,50,54).

4. Steelman et al. found that FSH preparations made by digestion with pancreatin were impure (44).

Steelman then set out to purify the material resulting from pan-

creatin digestion and following chromatography on DEAE cellulose, dialysis, lyophilization and rechromatography, produced a substance possessing 3000-4000 per cent of the activity of the Armour standard in the Steelman-Pohley assay (55); this substance showed no evidence of contamination by other hormones+ (51). However, in doses of 250 µg daily this preparation caused an increase in uterine weight in hypophysectomized rats, but when given daily for 21 days to hypophysectomized male rats it caused no increase in the weight of the ventral prostate. Steelman interpreted these findings as indicating that some estrogen secretion occurs under the influence of large doses of FSH. In his discussion of this paper, however, Greep expressed the view that the increase in uterine weight was due to contamination by ICSH and stated that some of his own earlier samples of swine FSH produced no change in uterine weight in large doses (56). Nevertheless, Steelman's preparation was homogeneous on DEAE cellulose, ultracentrifugation and paper electrophoresis. In contrast, undigested preparations purified in the same way were heterogeneous and unstable in the lyophilized state.

It is now possible to obtain highly active FSH and ICSH from sheep. The Koenig-King method yields a mixture of the two hormones. From this mixture Ward has shown that separation of the two gonadotrophins is readily effected by chromatography on carboxymethyl cellulose, eluting with 0.01 M phosphate buffer at pH 6.0; FSH leaves the column while ICSH is retained (34,35). This ICSH is purified to 100 per cent of the Armour standard (227-80) by gradient elution and the FSH is purified by DEAE cellulose as described above.

+ The basis for this statement is not given.

D. The Chemistry of Pituitary Gonadotrophic Hormones.

Steelman has studied the chemistry of his swine FSH purified after pancreatin digestion. The amino acid composition of this substance has been determined (51); FSH is high in aspartic and glutamic acids and in cystine-cysteine. The molecule also contains carbohydrates (galactose, mannose, fucose and hexosamine). Gurin originally showed that the percentage of hexose was approximately equal to that of hexosamine in the case of both ICSH and FSH (57). The discovery of fucose in FSH is the first example of the occurrence of this substance in a pituitary hormone, although it is found in mucoproteins elsewhere in the body.

The stability of FSH appears to be somewhat unusual in that preparations prepared without digestion are unstable in the lyophilized state even at -20°C, while preparations made by the same method following preliminary digestion with pancreatin are stable. Moreoever, 0.1 per cent cysteine causes the activity of digested FSH to decrease by a factor of 5, while 0.05 M Versene causes the activity of the same preparation to fall by a factor of 10 (51).

The chemistry of ICSH has been studied by Ward et al. who reported a high content of glucosamine (5.85 to 8.0 per cent depending upon the method and the preparation of LH examined) (34,35). Efforts to characterize the N-terminal amino acid of ICSH so far have not been successful (35).

A number of workers have shown that gonadotrophins differ chemically and immunologically from species to species. For example vanDyke and coworkers were able to demonstrate immunological differences between sheep and swine FSH (58), while Li and coworkers have compared ICSH

from the same two species as shown in Table II (22).

Table II

Comparison of the Chemical Properties of Sheep and Swine ICSH (after Li et al. (22))

Property	Sheep	Swine
Nitrogen (%)	14.2	14.93
Molecular Weight	40,000	100,000
Isoelectric Point	4.6	7.45
Mannose (%)	4.5	2.8
Hexosamine (%)	5.8	2.2

E. Assay of Gonadotrophic Hormones.

ICSH. Among the numerous methods used for the assay of relatively pure ICSH, the following appear at present to be the most important:

(1) increase in the weight of the ventral prostate

(2) ascorbic acid depletion of the ovary

(3) superovulation

(4) ovarian hyperemia

(5) weaver finch test.

Repair of interstitial cells of ovary or testis has been widely used in the past but such methods are tedious and time consuming, with the result that they have given way to more expedient procedures (4, 27,68-70).

(1) Ventral Prostate Method. The ventral prostate of the rat is an accessible and convenient end-organ for the estimation of the output

of testicular androgens and can consequently be used in the assay of ICSH. This method replaced that of Fevold, in which the weight of the seminal vesicles was measured (60), because the latter show a considerable difference in sensitivity from species to species. Apostolakis and Voigt have shown that the test is much more sensitive when the hypophysectomized immature rat is used (61). The method was devised originally by Greep and coworkers (62) and modified by Li et al. (63). Although the increase in weight of the ventral prostate is linear over a suitable dose range, the method has been criticized on the grounds that the response is affected by the presence of prolactin (64-66). Nevertheless, a method based on the assay of Li et al. has been used to measure ICSH in human urine (67).

(2) Ascorbic Acid Depletion. Parlow has shown that ICSH causes a fall in ovarian ascorbic acid and has developed an assay on the basis of this finding (71), analogous to that in current use for the measurement of ACTH. Normal adult rats 26-28 days old are prepared by subcutaneous injection of 75 I.U. of unfractionated pregnant mare plasma, followed 56-65 hours later by 25 I.U. of HCG in order to produce intense luteinization. The right ovary is removed for estimation of the control level of ascorbic acid 5-9 days after the injection of HCG. The material to be assayed is injected intravenously immediatly after removal of the right ovary and one hour later the second ovary is removed and assayed for ascorbic acid. The percentage decrease in ascorbic acid (maximal being 40 per cent) is proportional to the log of the dose of Armour standard LH between 0.125 and 1.0 µg. Other trophic hormones

are without effect in this assay. The method is quick and easy to perform so that it is rapidly gaining in popularity. So far, however, critical studies comparing this method with others have not appeared.

(3) Superovulation. The initiation of ovulation has been used as the basis for a number of methods for the assay of ICSH (72-79). Zarrow and coworkers have developed a method based upon the number of ova released following injection of ICSH in immature female rats pretreated with a sensitizing dose of pregnant mare serum (80). The authors claim that this method has a sensitivity 100 times that of the ventral prostate assay, but so far experience with this procedure has been limited.

(4) Ovarian Hyperemia. Two hours after injection of ICSH, the uptake of radioactivity by the ovaries of rats treated with radio-iodinated serum albumin is increased by as much as 110 per cent. This finding is the basis of the so-called hyperemia method of Ellis (81). The author claims freedom from interference by prolactin and FSH. It is at present too early to pass judgment on the value of this method.

(5) Weaver Finch Test. Within recent years a number of workers have used the response of the feathers of this bird to ICSH as a means of assaying the hormone (82,83). The end-point consists in the development of a black band in the feathers. FSH does not interfere with the test although human chorionic gonadotrophin (HCG) gives a positive response. The method is extremely sensitive (one unit being equivalent to 5 μg of Armour standard LH-227-80) but these small African birds appear to be difficult to raise in cold climates. At present, this method can be described as extremely promising; it

is mentioned chiefly because of the remarkable fact that this response of the feathers is extra-gonadal, being seen in castrated birds of either sex or in intact females (82,83).

Of these methods, that based upon the change in the weight of the ventral prostate of hypophysectomized rats appears to be the most reliable and best established. Ascorbic acid depletion is much simpler and may eventually replace rival methods.

FSH. Of numerous methods used for the assay of relatively pure FSH, the method of Steelman and Pohley (55) is at present the most popular. Methods such as the minimal dose required to re-establish follicular growth in hypophysectomized rats require elaborate histological methods (85-87). Methods used for the assay of mixed gonadotrophins (e.g., increase in ovarian weight (88-91), or in uterine weight (92-94) are of no interest in testing the biological purity of pituitary extracts since they are not sufficiently specific.

Steelman-Pohley Method (55). This method is based upon the observation that exogenous HCG increases the response of the immature rat ovary to FSH. Immature rats (21-22 days old) are pretreated with 20 I.U. of HCG subcutaneously on two consecutive days and 72 hours after the first injection, autopsy is performed and the ovaries are weighed. The increase in ovarian weight bears a linear relationship to the dose of FSH in the range of 0.05-0.3 mg of Armour standard FSH and the calculation of the data is based upon the slope ratio method of Finney (84).

F. The Action of Gonadotrophic Hormones.

Present knowledge of the action of gonadotrophins is based upon studies *in vivo* of the effects produced by injection of highly purified FSH and ICSH. Since neither hormone can be regarded as absolutely pure, the results of such experiments are not always unequivocal. It is only recently that the necessary techniques have been developed to explore the actions of these hormones *in vitro* so that present accounts of these actions are largely confined to gross morphological effects.

In 1942, Greep and Chow gave a detailed account of the biological effects of the FSH and ICSH which they had prepared from swine pituitary (27). Injection of ICSH into immature hypophysectomized male rats was shown to cause macroscopic enlargement of the testes and secondary sex organs, while microscopic examination revealed stimulation of the Leydig cells and seminiferous tubules. The authors further demonstrated that androgens were capable of repairing or maintaining the seminiferous tubules following hypophysectomy, but were without demonstrable effect upon the interstitial cells; they concluded that ICSH acts by stimulating the production of androgens by the interstitial cells. The level of ICSH nitrogen was shown to bear a quantitative relationship to the weight of the ventral lobe of the prostate, within certain limits and this observation gave rise to the method of assay already mentioned. Injection of ICSH into castrated hypophysectomized immature male rats was without effect on the secondary sex organs, indicating that adrenal androgens were not responsible for the original observations.

Injection of ICSH into immature hypophysectomized female rats

caused stimulation of the interstitial cells but no other detectable changes. In the normal adult female ICSH appeared to be unable to promote stimulation of the interstitial cells, which are presumably under maximal stimulation. However, ICSH could prevent or reverse the atrophy of the interstitial cells which follows hypophysectomy. Ovarian interstitial cells appeared to be much less sensitive to ICSH than the Leydig cells of the testis (27).

In immature hypophysectomized male rats, Greep and Chow showed that FSH increased testicular weight and produced microscopic evidence of tubular stimulation, without causing stimulation of the Leydig cells nor of the secondary sex organs (27). In immature female rats FSH produced maturation and growth of Graafian follicles, followed by atresia without either cyst formation or luteinization.

These findings have been amply confirmed and numerous details added to this general outline. For example, Li's group have shown that ICSH fails to restore tubular function and only slightly stimulates accessory genital structures if it is administered to hypophysectomized animals after a latent interval (95,96). On the other hand, small doses of ICSH, HCG or pregnant mare serum will maintain the seminiferous tubules following hypophysectomy if these preparations are administered immediately after operation (95). Larger doses are required to maintain the interstitial cells in a normal histological state and to maintain the accessory sexual apparatus (95). The same group also clearly indicated that interstitial cell-stimulation and luteinizing activity were properties of one molecule (21) and Greep showed that both activities were destroyed by trypsin (97).

Greep and coworkers showed that the effects of ICSH and FSH which they described in immature rats were also seen in hypophysectomized adult animals (98,99). Fevold et al. also indicated that ICSH does not act on immature ovaries (100) and together with Hisaw and Greep showed that copper salts stimulated the response in vivo to pituitary extracts (101). The response measured was increase in ovarian weight and the authors attributed their findings to the catalytic effect of copper salts upon the synergism between FSH and LH (123).

Many contributions have since been made to the details of gonadotrophic activity, especially in the female. In the male the general principle of two gonadotrophic activities has been repeatedly confirmed and will be used here as a starting point for chemical investigations of the mechanism of these activities.

G. Chorionic Gonadotrophin.

1. Discovery In 1928, Aschheim and Zondek discovered a gonadotrophic substance in the blood and urine of pregnant women which they named Prolan, in the mistaken belief that it was of pituitary origin (102). This substance was detected by the maturation which it caused in the ovaries of immature rats and mice. The extra-pituitary origin of this substance was established by a number of observations including (4):

(1) Failure to find increased gonadotrophic activity in the pituitary during pregnancy.

(2) Certain qualitative differences were noticed between the new gonadotrophin and those found in the blood and urine of castrate humans - the latter showing more FSH-like activity. Again, pituitary

gonadotrophins were shown to cause a greater increase in ovarian weight in immature rodents if injected over a period of 72 to 96 hours. In this test increasing doses of the new gonadotrophin caused a maximal response which was considerably less than that produced by pituitary extracts.

(3) Prolan produces a localized crop of follicles rather than the larger number seen following the administration of FSH.

(4) In hypophysectomized rodents Prolan causes no follicular stimulation, its action being confined to stimulation of the interstitial cells.

These differences led to a search for the source of the new hormone and since its appearance in blood and urine coincided with the development of the placenta, this tissue was regarded as a likely source. In 1930, Collip confirmed this idea by studies with placental implants and extracts (103). Seven years later Kido showed by means of placental implants in the anterior chamber of the rabbit eye that the placenta was the site of secretion of the hormone and not merely a storage depot (104). Tissue cultures showed that the Langerhans cells secreted the placental gonadotrophin (105,106), which was appropriately called chorionic gonadotrophin.

2. Purification and Present Preparations - The major source of chorionic gonadotrophin is human pregnancy urine and preparations from this source are called human chorionic gonadotrophin, abbreviated to HCG.

In 1940, Gurin and colleagues obtained a highly purified form of HCG from the urine of pregnant women by adsorption on benzoic acid, ethanol fractionation and extraction with chloroform (107,108). This

material showed an activity of 6,000-8,000 I.U. per mg and appeared "nearly homogeneous" on electrophoresis. Katzman and colleagues achieved the same biological potency by chromatographic adsorption on permutit followed by elution with 10 per cent ammonium acetate in aqueous ethanol (109).

In 1948, Claesson et al. succeeded in crystallizing the hormone by precipitation with protamine; their preparation also showed an activity of 6,000-8,000 I.U. per mg (110).

In 1953, Lyon et al. showed that pregnancy urine contained FSH (111), which indicated a possible source of contamination of HCG extracts. Furthermore, Morris and Morris have obtained the most active preparation so far reported (112); this is homogeneous by electrophoresis but not by ultracentrifugation or by solubility studies. It is therefore likely that highly purified preparations of HCG have yet to be achieved. Commercially available preparations of HCG are made by a process described by Butt (113). This involves the combined use of sodium benzoate and sodium tungstate.

HCG is a protein, soluble in water, in 50 per cent acetone and in 50 per cent alcohol. The physico-chemical properties of Gurin's preparation are shown in Table III where it will be seen that HCG is a glycoprotein and that the hexose content is approximately twice that of hexosamine, whereas in the case of FSH and LH the content of hexose and hexosamine are nearly equal (59). The hormone is inactivated by trypsin and chymotrypsin and by salivary enzymes (114) (presumably indicating that the carbohydrate moiety is essential for activity). Wettstein and Benz have shown that smaller peptide fractions of HCG resulting from pepsin hydrolysis are biologically active

(115).

Gurin et al. showed that the amino group of the hexosamine is acetylated and that another acetyl group is present elsewhere in the HCG molecule (107). They regard the ratio of hexose to hexosamine as suggesting that the molecule is built up of hexosamine digalactose units.

Table III

Physico-chemical Characteristics of Human Chorionic Gonadotrophin (Cohen (59))

Component	Per cent
Carbon	50.06
Hydrogen	7.03
Nitrogen	12.35
Total Reducing Sugar	16.0
Galactose	11.8
Hexosamine	6.32
Moleuclar Weight	100,000
Isoelectric Point	3.2-3.3

3. Action - In the female, HCG stimulates follicular development, increases the weight of the immature ovary, stimulates the interstitial cells and renders the ovary much more sensitive to the action of FSH. As mentioned previously, HCG does not stimulate follicular development in hypophysectomized animals, although it increases the sensitivity of the ovaries to FSH in such animals (117,118). Moreover HCG has been shown to act synergistically with subthreshold doses of pituitary extracts, the mixture producing a response greater than the sum of the responses to the two extracts

administered separately (59,110,117,118). These findings have suggested that HCG stimulates the release of FSH from the pituitary (118,119) while it also exerts a direct effect upon the interstitial cells. If this is so, the most important difference between the action of HCG and that of ICSH may lie in the capacity of HCG to bring about the release of pituitary FSH.

In the human, Claesson et al. showed that intravenous injection of HCG was followed by follicular stimulation and increased estrogen production (110). Such follicular stimulation was followed by atresia, although if small doses of pregnant mare gonadotrophin were added, rupture of follicles and formation of corpora lutea took place.

In the male, chorionic gonadotrophin appears to exert a predominantly interstitial cell-stimulating effect although some stimulation of the germinal epithelium does occur (119,121). Whether the latter is due to contamination with FSH or represents a response to androgens secreted by the Leydig cells or the release of pituitary FSH or a direct action of HCG itself, cannot be stated at present. Chorionic gonadotrophin also promotes the secretion of estrogens by the testis (119) and in large doses produces destruction of germinal tissue (122).

A clear statement of the action of HCG must await the development of more highly purified preparations. At present the hormone is regarded as producing direct stimulation of interstitial cells and as causing release of FSH with which it acts synergistically. The significance of HCG in the present work lies in its use as a cheap and available source of an interstitial cell-stimulating hormone to be used in pilot studies preparatory to the use of the more

expensive ICSH.

One curious distinction between chorionic gonadotrophin and ICSH is that the latter injected intraperitoneally antagonizes other gonadotrophins simultaneously injected subcutaneously. This antagonism is not seen with HCG nor when ICSH is injected subcutaneously, nor in hypophysectomized animals (4).

4. Assay - It was decided in 1938 at the Third International Conference on the Standardization of Hormones, to establish an international standard for the gonadotrophic material of human urine of pregnancy (120). An international unit was defined as follows:

> "The specific gonadotrophic activity of 0.1 mg of the standard preparation shall be the international unit for the activities of all gonadotropic preparations of human urine of pregnancy, but only of such."

It was further agreed that the only tests to be recommended for the comparative assay of gonadotrophic activity in terms of this unit were those depending upon:

> "a. the observation of a direct or indirect gonadotropic effect shown by morphological changes in the gonads or
> b. the observation of secondary changes in the accessory reproductive organs in animals not deprived of their gonads."

The provision was made that in the case of (b), control studies be used to exclude direct action upon the accessory reproductive organs, using castrate animals.

Among the methods which conform to these requirements, the following have been used:

(1) Increase in uterine weight in rats.

(2) Increase in ovarian weight in rats.

(3) Vaginal cornification in rats.

(4) Corpus luteum formation in mice.

(5) Ovulation in rabbits.

(6) Repair of ovarian "deficiency cells" in hypophysectomized immature rats.

(7) Increase in ventral prostate weight in rats.

The most widely used of these methods are (1), (2) and (7). Apostolakis and Voigt have shown that increase in ventral prostate weight in intact rats provides the basis of an accruate method, while increase in ovarian weight is less satisfactory (61). A method described by Dorfman and Rubin using the weight increase of the uterus of the immature rat as a measure of activity has proved valuable (116).

H. The Mechanism of Action of Trophic Hormones.

1. Gonadotrophins - The fact that the two gonadotrophins act upon the gonads and that they control both the germinal and endocrine functions of these organs, together with the complex cyclic activity of female reproduction, contributes to the difficulties of studying the mechanism of gonadotrophic activity. Moreover, highly purified gonadotrophins have not been freely available, so that progress in this field has been slower than in the case, for example, of the mechanism of action of ACTH. Since HCG has been freely available from human pregnancy urine, early studies were made with this hormone in the belief that it acts in a similar manner to ICSH.

In 1952, Brady attempted to show that HCG stimulates the rate of incorporation of labeled acetate into testosterone by slices of testis in vitro (124). It is probable that Brady succeeded in this

undertaking, since his findings have been confirmed (see page 116), but in the original paper the author failed to establish the identity of the radioactive compound extracted from the incubation medium. Radioactivity was measured by eluting the carrier testosterone (located by ultraviolet absorption) from paper chromatograms, followed by plating on aluminum planchettes which were counted in a windowless Geiger counter.

Brady used testicular tissue from hog, rabbit and man, although stimulation by HCG was only reported in the case of rabbit testis. The author showed an approximately ten-fold increase in the radioactivity associated with carrier testosterone in the case of stimulated testis. The isolation of testosterone depended upon column chromatography and one system of paper chromatography; no derivatives were prepared and recrystallization to constant specific activity was not reported.

Brady also attempted to measure the amount of radioactive cholesterol produced in these experiments by means of digitonin precipitation. In the presence of HCG the rate of incorporation of acetate into testosterone was increased some ten-fold but no significant change in the specific activity of cholesterol was observed. These findings have been regarded as evidence against cholesterol as an obligatory precursor in the biosynthesis of testosterone although they are open to a more probable interpretation (see page 50).

In 1956, Samuels and Helmreich showed that HCG increases the production of the enzyme steroid-3β-ol-dehydrogenase in the testis (125). This increase occurred only 12 to 24 hours after administration of HCG and was accompanied by increase in testicular weight. The authors

accordingly suggested that the increase in production of this enzyme may merely reflect a non-specific increase in testicular protein.

In the following year, Brinck-Johnson and ik-Nes showed that HCG stimulates the production of testosterone and of Δ^4-androstene-3, 17-dione in the dog in vivo* (126). Following a single intravenous injection of the hormone, elevated levels of these two steroids were maintained in spermatic vein blood for several hours. The data did not enable the authors to define the mechanism of this stimulation, although the rapidity of the observed response was taken to favor the idea of stimulation of an enzyme already present in the tissues rather than increased production of an enzyme present in limiting concentrations. Perhaps more recent experience with protein synthesis in vitro makes it necessary to leave this question open, since measurable incorporation of labeled amino acids into protein occurs within a few minutes in some tissues (129).

Studies with ICSH in the rat ovary have shown that this hormone causes a fall in ovarian ascorbic acid and cholesterol (128). Both substances fall within one hour of an intravenous injection of ICSH and have returned to normal levels within 24 hours of a single injection.

Studies with FSH have been fewer. In 1958, the Hollanders showed that a preparation of FSH made by Dr. S. L. Steelman stimulated the conversion of testosterone-4-C^{14} to estradiol-4-C^{14} by slices of dog ovary in vitro**, whether the hormone was administered in vivo

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* Later in the same year Hollander and Hollander repeated these findings (127).

** Unfortunately the possible effect of contaminating ICSH cannot be excluded from these experiments in view of a personal communication from Dr. Steelman (page).

or *in vitro* (130). Administration *in vivo* was not followed by further stimulation when the hormone was added *in vitro*. HCG exerted the same effect and both hormones acted on the anestrous ovary. The authors were led to the following conclusions:

(1) Aromatization of testosterone may be a step in the physiological path to estradiol.

(2) FSH stimulates a rate-limiting step in the conversion of testosterone to estradiol.

(3) The prompt response observed is unlikely to result from increased "enzyme synthesis due to growth ".

The first of these conclusions cannot be opposed in view of the cautious wording used. That testosterone may be converted to estradiol does not mean that it is or that this step is obligatory. The second conclusion is probable but should be phrased to embrace the possibility that FSH may make some essential cofactor (e.g., TPNH) or some source of energy available for the process of aromatization. Recent studies in other organs indicate that the third conclusion is unjustified, since protein synthesis is more rapid than was formerly believed (129). The data presented do not exclude the possible effect of contaminating ICSH (see page 152).

One further piece of data concerning the action of gonadotrophic hormones has been provided by Eik-Nes *et al.*, namely that FSH, ICSH and ACTH do not cause ascorbic acid depletion in the testis (131).

2. Corticotrophin - Since the adrenal cortex proved to be highly active *in vitro* using perfusion, slices and homogenates, since the products of its synthetic activity have been carefully characterized and since stimulation *in vitro* with ACTH is readily observed

in the case of perfusion and that of slices, it is only to be expected that the response of this tissue to ACTH proved much more informative that that of the sex glands to gonadorophins.

Perfusion Studies. The first step towards an understanding of the action of ACTH came from the perfusion studies of Hechter and his colleagues (132). The authors showed that the production of corticosteroids by the perfused adrenal without added substrate proceeded extremely slowly but at a considerably greater rate in the presence of ACTH. Moreover, 11-hydroxylation did not require the presence of ACTH to proceed at a significant rate and both pregnenolone and progesterone were converted to cortisol without ACTH. This seemed to indicate that ACTH does not stimulate the biosynthetic pathway after pregnenolone. Moreover, the authors produced evidence for the view that the step between pregnenolone and its natural precursor is the biosynthetic pacemaker, even in the presence of ACTH. Since it now seems likely that this precursor is cholesterol, it is assumed that the conversion of cholesterol to pregnenolone may be specifically sensitive to ACTH, although proof of this view with slices or homogenate is still awaited.

Increasing experience with the use of ACTH <u>in vitro</u> indicated that the hormone was highly active in stimulating the production of corticosteroids by slices but great difficulty was experienced in achieving stimulation of homogenates; in fact, it is generally believed that the hormone is inactive in subcellular preparations (113,134,138). Meanwhile, it was shown that ACTH, while stimulating steroid production depletes the cortex of cholesterol (132,135,136) and of ascorbic acid (149-154). The fall in adrenal cholesterol is

consistent with the data of Hechter et al. (132) and presumably reflects the use of cholesterol as a substrate for steroid synthesis.

Reduced Pyridine Nucleotides. In 1957, the first of a series of papers appeared which offered promise of clarifying the mechanism of action of ACTH. Haynes and Berthet studied the problem from three aspects (137):

(1) The authors showed that ACTH stimulated synthesis de novo of corticosteroids and not merely their release from adrenal cells.

(2) The influence of reduced TPN, and of systems which generate reduced TPN, upon the production of corticosteroids was studied.

(3) The influence of various carbohydrates upon the production of corticosteroids was studied.

The first point was clearly and convincingly demonstrated, while the second approach gave results shown in Table IV.

Table IV

The Influence of Reduced TPN Upon the Output of Corticosteroids by Adrenal Homogenate (After Haynes and Berthet (137))

Addition	Output of corticosteroids µg per 30 minutes
None	2
Fumarate 4×10^{-2}M	99
Fumarate 4×10^{-2}M; TPN 4×10^{-4}M	136
TPN 4×10^{-4}M	2
TPNH 6×10^{-3}M	22

These data indicate that:

(1) The adrenal homogenate is not stimulated by TPN.

(2) Fumarate causes a considerable increase in corticosteroid output.

(3) Fumarate and TPN together cause a still greater response.

(4) TPNH exerts, in comparison with (2) and (3), a modest degree of stimulation.

The authors regarded these findings as indicating a specific dependence of steroid production upon the available supply of reduced TPN. However, certain objections to this claim might be considered.

(1) Addition of a high concentration of TPNH produces a relatively small response in comparison with fumarate.

(2) The findings would be more convincing if a dose-response curve were available. The data of Glock and McLean indicate that rat adrenal contains a total concentration of TPN (reduced and oxidized) of 1.33×10^{-4} mg per mg of tissue (217). The dose used in these experiments provides approximately 10^{-2} mg per mg and it is unfortunate that the only concentration of TPN reported is 10 times less than that of TPNH.

(3) The design of these experiments could be improved if the authors had not fixed upon the arbitrary time of 30 minutes; one is interested in the rate of production of steroid and a time response curve would be valuable.

(4) The device of adding fumarate follows from the findings of Reich and Lehninger (246) and is believed to act by stimulating transhydrogenase activity, as shown for steroid hydroxylase activity by Sweat and Lipscombe (247). If this is the mechanism by which fumarate causes stimulation of corticosteroid activity in the present system, studies of the influence of fumarate with DPNH seem indicated.

The third approach gave the data shown in Table V.

Table V

The Influence of Various Carbohydrates Upon the Production of Corticosteroids by the Adrenal Gland (After Haynes and Berthet (137))

TPN 4×10^{-4}M in each flask		Corticosteroid Output µg/30 min.	
Addition		Homogenate from control slices	Homogenate from ACTH treated slices
None		2	3
G-6-PO_4	10^{-2}M	110	102
G-1-PO_4	10^{-2}M	91	87
Glycogen	10 mgm/ml	5	14
Glycogen	+ liver phosphorylase	69	62

These findings reveal that:

(1) Glucose-1-PO_4 is almost as potent as glucose-6-PO_4 in stimulating corticosteroid production and pretreatment with ACTH does not enhance the response in either case.

(2) Glycogen causes slight stimulation but greater stimulation following exposure to ACTH. Addition of liver phosphorylase with glycogen causes a considerably greater response.

Haynes and Berthet conclude that ACTH stimulates steroid production by increasing the available supply of reduced TPN. Again, however, their data raise several problems. The response to ACTH in the presence of added TPN is disappointing and does not favor the interpretation that this hormone is specifically capable of promoting reduction of TPN. The excellent response to the added hexose phosphates and the failure of ACTH to enhance this response might mean

that the cortex has been depleted of glycogen by a response to stress before the experiment (e.g., during handling before sacrifice).

The following year brought a paper from Koritz and Péron (138) and a second paper by Haynes (139). Koritz and Péron (138) confirmed the stimulating influence of reduced TPN which produced a 3-to 7-fold rise in corticosteroid production. However, this effect was further enhanced by ACTH and the maximal response to ACTH was further increased by addition of TPN and a reducing system. Freezing prevented the response of slices to ACTH (134) but allowed a response to TPN and a reducing system which exceeded the combined effect of ACTH and reduced TPN in non-frozen slices. The combined effect of ACTH and reduced TPN was not additive.

The authors believe that their data suggest a two-fold action of ACTH, namely production of reduced TPN and enhanced synthesis of corticosteroid precursors. They regard the action of freezing as leading to the release of precursors from some bound or unavailable state and suggest that homogenization may have a similar effect, thus explaining the difficulty of demonstrating ACTH stimulation in a homogenate (133,134).

Haynes' second paper convincingly demonstrated that ACTH increases the adrenal content of cyclic AMP and that this increase is associated with stimulation of adrenal phosphorylase activity (139). It was not possible to demonstrate this increased production of cyclic AMP in a homogenate. Haynes showed that the response of the cortex was specific to the extent that ACTH had no effect on liver phosphorylase, while epinephrine and glucagon had no effect upon adrenal phosphorylase.

In 1959, Haynes, Koritz, and Péron proceeded to study the influence of added cyclic AMP upon adrenal slices (140). The authors made the following observations:

(1) Cyclic AMP stimulated the production of corticosteroids.

(2) Cyclic AMP can stimulate the cortex when this has been maximally stimulated by ACTH.

(3) 10 micromoles of cyclic AMP cause greater stimulation than the maximal response to ACTH.

(4) The response to cyclic AMP is specific in that a number of related nucleotides proved to be without effect.

(5) Cyclic AMP failed to stimulate an adrenal homogenate which was capable of producing corticosterone.

The authors claim that these results place "cyclic AMP as an intermediate in the chain of events leading to corticosteroid production".

These papers taken as a whole establish a response to ACTH which takes the form of enhanced phosphorylase activity and strongly suggest that this response is mediated by increased production of cyclic AMP which, in the case of the liver, is known to influence the equilibrium

$$\underset{\text{[inactive]}}{\text{Phosphorylase b}} \rightleftharpoons \underset{\text{[active]}}{\text{Phosphorylase a}}$$

in favor of phosphorylase a. Furthermore, with a generous addition of reduced TPN, an adrenal homogenate produces more corticosteroids in 30 minutes than a control homogenate. We are asked to consider the extrapolation that ACTH promotes steroid synthesis by increasing

the activity of adrenal phosphorylase which leads to enhanced glycogenolysis and disposal of the resulting glucose-6-PO_4 by way of the pentose phosphate pathway in amounts sufficient to produce greater quantities of TPNH than are found in the unstimulated gland.

Before this theory can be accepted, four major difficulties need to be resolved:

(1) Can it be shown that in the adrenal cortex, stimulation of phosphorylase activity increases the proportion of endogenous TPN in the reduced form?

(2) Can it be shown that in the adrenal cortex the availability of reduced TPN is a limiting factor in steroid synthesis?

(3) Why does cyclic AMP not act in a homogenate?

(4) Can reduced TPN cause a prolonged stimulation of corticosteroid production or is its activity confined to acute bursts of stimulation which consume a waiting supply of available precursors?

Solution to these problems may come from the unequivocal demonstration that glycogenolysis necessarily produces increased disposal of glucose-6-PO_4 by way of the pentose phosphate pathway or by proof that ACTH causes a shift in the ratio of oxidized to reduced TPN in the endogenous pyridine nucleotide of the cortex, as distinct from TPNH added to incubation media as a source of stimulation. The solution to the first problem has so far proved elusive and the ingenious methods employing isotopically labeled carbohydrates (224-229) require assumptions which have yet to be justified. On the other hand, the solution of the second problem may well hinge upon the development of methods which will allow estimation of both oxidized and reduced forms of both pyridine nucleotides in such concentrations

as are found within adrenal cells. In this connection the findings of Glock and McLean are not encouraging (217). Table VI shows the data obtained by applying a method which they devised.

Table VI

Levels of Oxidized and Reduced Coenzymes in Animal Tissues (after Glock and McLean (217))

Tissue	Coenzyme Content (μg/g tissue)					
	DPN^+	DPNH	DPN^++DPNH	TPN^+	TPNH	TPN^++TPNH
Rat adrenal (5)†	315±136	154±45	469±134	17±9	116±24	133±24
Rabbit adrenal (1)	295	117	412	<2	62	62
Rabbit adrenal cortex (1)	356	133	489	14	68	82
Rabbit ovary (1)	181	34	215	<2	42	42
Rat testis (1)	80	71	151	<2	6	6

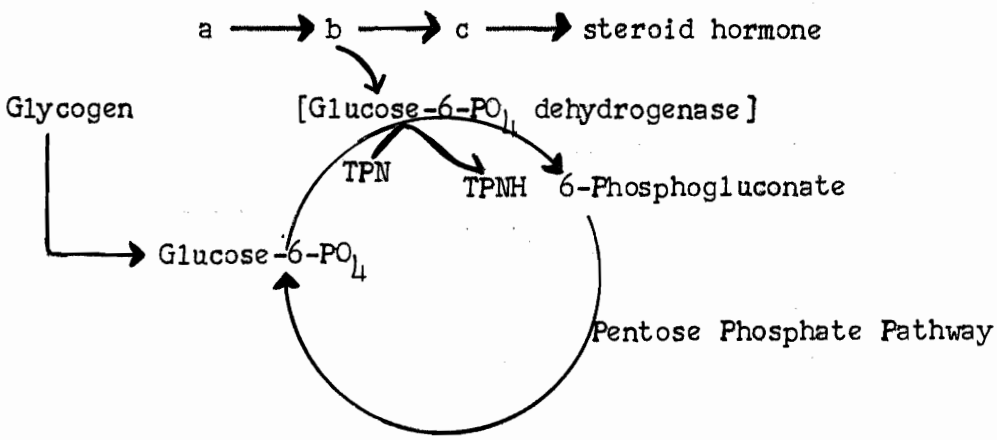
† Figures in parentheses represent the number of animals.

In the case of the adrenal most of the TPN appears to be reduced and if these animals can be regarded as unstimulated, the measurement of increase in the reduced form may prove extremely difficult.

However, Holzer has indicated that the more recent methods show that the ratio DPNH/DPN in several tissues is of the order of 1:1000 (248) whereas previous workers (249) had obtained values 1:2 to 1:5 by using methods such as that of Glock and McLean in which cells are destroyed by acid, alkali or heat. It may, therefore, be wise to assume that the state of intracellular TPN is not known at present.

Haynes' findings may possibly be interpreted in a different way. Marks and Banks have shown that a number of steroids are capable of inhibiting glucose-6-PO_4 dehydrogenase (141). This inhibi-

tion is seen with low concentrations of steroid (10^{-6}M) and inhibition of spinach or yeast glucose-6-PO_4 dehydrogenase is not produced by these steroids even at higher concentrations. Dehydroepiandrosterone and pregnenolone were particularly potent inhibitors, while steroid hormones such as cortisol, estradiol and testosterone were less effective. It may be suggested that this effect could be relevant to the rate of production of TPNH. If the synthetic pathway to steroid hormones is considered, some precursor could inhibit glucose-6-PO_4 dehydrogenase thus:



b is regarded as inhibiting glucose-6-PO_4 dehydrogenase and hence checking the rate of production of reduced TPN. Stimulation by ACTH could lower existing levels of b and hence decrease this inhibitory effect. In this way coupling would be achieved between ACTH stimulation and the production of an essential cofactor (TPNH). The consequent depletion of glucose-6-PO_4 may stimulate glycogenolysis; this response may involve cyclic AMP in some manner other than by a direct effect of ACTH upon the production of this nucleotide.

In this connection, a recent publication by Rose provides evidence that a rate-limiting step in glucose oxidation by human red

cells and ascites tumor cells exists beyond glucose-6-phosphate dehydrogenase and may involve 6-phosphogluconate dehydrogenase (254). If this is true of adernal tissue, the mechanism postulated above may apply to 6-phosphogluconate dehydrogenase rather than to glucose-6-phosphate dehydrogenase.

The hypothesis that ACTH acts by stimulating the production of cyclic AMP and hence by promoting phosphorylase activity, is especially attractive in view of the well known action of glucagon and epinephrine, which exert the same effect upon the production of cyclic AMP in the liver, while epinephrine does this also in muscle (142-146). An attempt has been made to apply this hypothesis to the effect of ICSH upon the ovary. Marsh and colleagues showed that the rate of incorporation of acetate into progesterone by slices of beef corpus luteum is stimulated 3 to 12 fold by addition of TPN (with or without a TPN-reducing system) but not by DPN (147). These findings point to a stimulating effect due to an increase in total concentration of triphosphopyridine nucleotide, regardless of its state of oxidation and do not suggest a specific requirement for reduction such as that which operates by way of cyclic AMP. This response in ovarian slices may be nonspecific and may be related to changes in permeability of the large, active cells of luteal tissue.

Protein Synthesis. Since ACTH causes an increase in the weight of the adrenal cortex, accompanied by histological changes suggestive of protein synthesis, Koritz et al. tested the hypothesis that corticotrophin stimulates enzyme synthesis (148). They studied the rate of incorporation of glycine-1-C^{14} and phenylalanine-C^{14} into protein by stimulated and unstimulated adrenal slices. The authors showed that

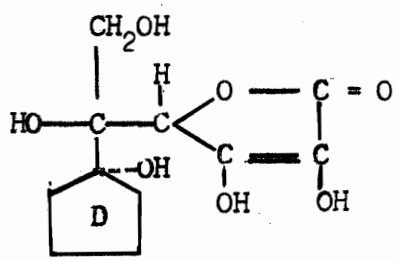
slices stimulated by corticotrophin showed increased steroid production without demonstrable change in the rate of incorporation of these amino acids into material precipitated by trichloracetic acid. These results do not exclude protein synthesis as a possible response to ACTH, since a small but significant stimulation of amino acid incorporation into one specific protein might be overlooked by such a method. More detailed studies of microsomal protein may prove more sensitive than whole cell material. Furthermore, it is difficult to select two amino acids which may best reflect small changes. Glycine undergoes a complex metabolic disposal, including nucleotide synthesis and complete removal of nucleotides from a trichloracetic acid precipitate may be difficult. It may also happen that the protein of which the synthesis is being studied may be low in phenylalanine.

Ascorbic Acid Depletion. The stimulating influence of ACTH upon the cortex is associated with the phenomenon of ascorbic acid depletion (136,149-154), which remains one of the intriguing mysteries of adrenal physiology. The fact that such depletion is quantitatively related to the dose of ACTH suggests that this change in ascorbic acid content might be intimately related to the mechanism of action of ACTH. Experiments <u>in vitro</u> by Slusher and Roberts showed that ascorbic acid depletion was almost complete within 15 minutes of exposure to ACTH, whereas increased corticosteroid production was scarcely evident at that time (149). Among the explanations offered to account for the phenomenon of ascorbic acid depletion are the following:

(1) Schmidt and colleagues proposed that ascorbic acid may play a role in the biosynthesis of corticosteroids (150).

(2) Hechter et al. (151) suggested that ascorbic acid may be responsible for maintaining cofactors used in steroid biosynthesis in an appropriate state of reduction (151).

(3) Loewenstein and Zwemer isolated a steroid from adrenal tissue, which appeared to be bound through C_{20} to ascorbic acid (152). They postulated the following structure:



and suggested thatincreased corticosteroid production involved release of steroids from the gland in such a bound form. So far, this finding does not appear to have been repeated.

(4) Ascorbic acid may not be related to the biosynthesis of corticosteroids and depletion may be an expression of increased permeability of adrenal cells, which is one suggested mechanism for the action of ACTH (157).

(5) It remains possible that ACTH causes the release of ascorbic acid from the cortex into the blood stream and that ascorbic acid acts at some extra adrenal site. So far this possibility appears to have escaped formal statement.

(6) Hayano and colleagues have expressed the belief that ascorbic acid may act as a brake on steroid synthesis, the release

of which is accompanied by increased production of corticosteroids (153).

Slusher and Roberts realized the need for examining the fate of ascorbic acid soon after administration of ACTH and in this way succeeded in showing that loss of adrenal ascorbic acid was accounted for by the rise in ascorbic acid content of the venous effluent (149). This finding excludes the first possiblity considered above and makes the second improbable since, if ascorbic acid were to discharge a protective function upon necessary cofactors, it would be expected to persist in the gland and to suffer a reversible oxidation, acting to maintain a reductive environment in which TPN, for example, is reduced as fast as it is oxidized during steroid synthesis. It may be helpful to emphasize this important finding of Slusher and Roberts by speaking of ascorbic acid discharge rather than depletion.

The findings that scurvy is associated with an initial rise in the adrenal content of cholesterol (250) and in the plasma 17-hydroxy-corticosteroid concentration (155) suggest that a direct and intimate relationship between the level of adrenal ascorbic acid and steroid synthesis is lacking. The changes in adrenal cholesterol reported by Oesterling and Long were found in scorbutic guinea pigs in which the adrenal ascorbic acid was reduced from 164.7±7.0 to 6.02±2.0 (250).

Against the third view has been leveled the finding of Vogt that corticosteroids are not released from the adrenal bound to ascorbic acid (154). Moreover, Slusher and Roberts found that loss of ascorbic acid precedes release of steroids. However, these arguments do not exclude the possibility that steroids are stored in bound form and the first step in their mobilization consists of

release of bound ascorbic acid not only from the steroids but from the gland. Nevertheless, the fact that the findings of Lowenstein and Zwemer have not been repeated makes this possibility largely conjectural.

The incidental release of ascorbic acid following increased cell permeability produced by ACTH is based upon a naive concept of membrane transport. It is not likely that changes in cell permeability represent a non-specific opening of "flood-gates" which allows the release of cell contents at random. It is now believed that transport of substances across cell membranes is a deliberate and specific process and if ACTH were to affect this process, it would seem logical to expect the hormone to act by encouraging the accumulation of factors needed for steroid synthesis within the cell.

The view that ascorbic acid inhibits corticosteroid production is based upon studies of the specific activities of cholesterol-4-C^{14} and those of steroids produced from this substrate by mitochondria, in the presence and absence of ascorbic acid (153). Hayano et al. showed that ascorbic acid inhibited the conversion of cholesterol-4-C^{14} to progesterone and they could explain their findings by assuming that the vitamin caused the exclusion of some 40 per cent of the mitochondrial cholesterol from the enzymatic pathway to corticosteroids (153). In many ways this hypothesis offers the most satisfactory solution to the problem, although more detailed studies of time and concentration of ascorbic acid are needed. Corticotrophin would then act by removing this inhibitory effect of ascorbic acid. The observations of Llaurado and Eik-Nes would suggest that ICSH does not act by the same mechanism (131).

The suggestion that ascorbic acid is released from the cortex to discharge some extraadrenal role remains purely hypothetical.

Harding, Rutherford and Nelson have recently shown that ACTH causes the appearance of oxidized ascorbic acid in the adrenal vein of the rat (156). Their findings indicate that soon after the administration of ACTH adrenal ascorbic acid is oxidized and discharged into the adrenal vein. These observations suggest that the vitamin is used in the capacity of a reducing agent and is then released. Whether ascorbic acid is concerned with the reduction of TPN or with the maintenance of reduced SH groups in the enzymes concerned with protein synthesis, is not likely to be revealed until a clearer understanding is available of the role of ascorbic acid in the oxidation-reduction systems of tissues. It may also turn out that ascorbic acid acts within a particular subcellular compartment.

The depletion of ascorbic acid observed in the ovary following the administration of ICSH is a much slower change (128) and so far the fate of this ascorbic acid has not been explored.

Cell Permeability. Since evidence has been produced to show that insulin promotes cell permeability to glucose, Eichhorn and coworkers tested the influence of ACTH upon the permeability of adrenal cells to sugars (157). They showed that hypophysectomy was followed by a decrease in the distribution of D-xylose in the intracellular water of the adrenal glands and that ACTH caused an increase in the entry of this substance, specifically into adrenal cells; the resulting levels approached those seen in intact animals subjected to stress.

Subsequent studies in the same laboratory showed that the behavior of adrenal tissue *in vitro* was quite different (158). Bisected rat adrenals allow rapid penetration of D-xylose and equilibration with approximately 70 per cent of the cell water; ACTH does not influence this behavior under conditions in which it stimulates corticosteroid production. These findings seem to suggest that the stimulating influence of ACTH on steroid biosynthesis does not depend upon the regulation of cell permeability to sugar. However, the transport of sugars in the case of the adrenal may involve a different specificity from that seen in the case of insulin. The failure of ACTH to influence penetration of D-xylose *in vitro* is presumably due to the free penetration seen in the unstimulated tissue, which in turn may reflect tissue damage in the preparation of slices, as the authors suggest (158). Another possibility is that the exclusion of D-xylose seen *in vivo* represents a vascular phenomenon which is altered following hypophysectomy by some non-specific mechanism.

II. THE BIOSYNTHESIS OF STEROID HORMONES

A. Biosynthetic Pathways.

Studies in vitro indicate that all steroid-forming tissues can convert acetate to steroid hormones and the two carbon atom fragment is generally regarded as the fundamental synthetic unit of steroid hormones (124,159-162). Activation of acetate to acetyl CoA and its condensation with acetoacetyl CoA produces β-hydroxy-β-methylglutaryl CoA and this, in turn, is reduced to mevalonic acid. The pyrophosphate of mevalonic acid yields two isomeric five carbon atom pyrophosphates which condense to produce the triterpene squalene (163-165) (Fig. 1).

Tchen and Bloch used hog liver homogenate to study the fate of squalene since this system converted squalene to lanosterol and not to cholesterol (166,167). The authors found that when this conversion was performed in the presence of D_2O, no deuterium was found in the lanosterol, which suggested that partly cyclized substances were not formed as intermediates. Moreover, the cyclization of squalene required molecular oxygen and did not incorporate O^{18} from H_2O^{18} (167). It is now clear that hydroxylation of squalene at the carbon atom which is to become C_3 of cholesterol, promotes a series of electron shifts associated with the opening of double bonds and ring fusion (167,169). These changes are accompanied by two 1,2-methyl shifts to give lanosterol, according to

the scheme proposed by Woodward and Bloch in 1953 (168,169). In spite of the low specific activity of squalene in experiments in which cholesterol is synthesized from labeled acetate, the present evidence is entirely in keeping with squalene as a major, if not an obligatory, precursor of cholesterol, since only a fraction of the squalene in sterol-forming organs is involved in sterol synthesis and a large inert pool of squalene accounts for the low specific activity of this compound in such experiments (170).

The conversion of lanosterol to cholesterol involves

(1) demethylation,

(2) oxidation and subsequent reduction of the hydroxyl group at C_3,

(3) rearrangement of double bonds,

(4) reduction of the Δ^{24} double bond in the side-chain of desmosterol (Fig. 2).

The exact sequence of these steps and the nature of all the intermediate compounds formed is not yet certain.

It has not so far been possible to establish the proposal that cholesterol is an obligatory precursor of steroids (181). Nevertheless, within an error of 8 per cent, all of the C_{21} of cortisol comes from C_2 of acetate and all of the C_{20} from C_1 of acetate (182,183). Furthermore, 10 carbon atoms of androstenedione and 9 of estrone come from C_2 of acetate. These findings are compatible with the folding of squalene to form cholesterol or some substance closely resembling cholesterol in structure. Finally, cholesterol has been shown to serve as a precursor for all the known steroid hormones (184,185). Evidence against cholesterol as an obligatory

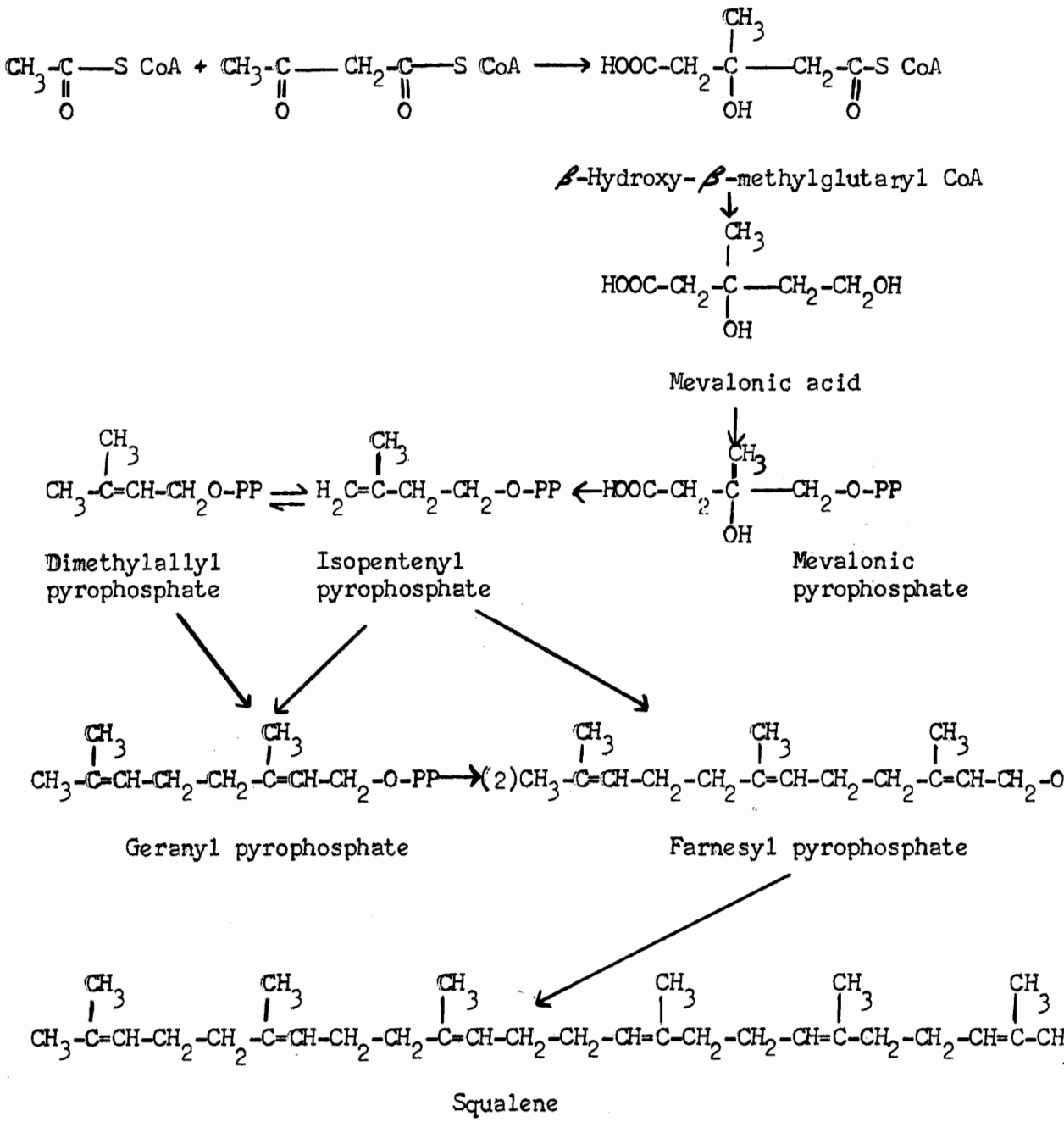


Figure 1. The biosynthesis of squalene

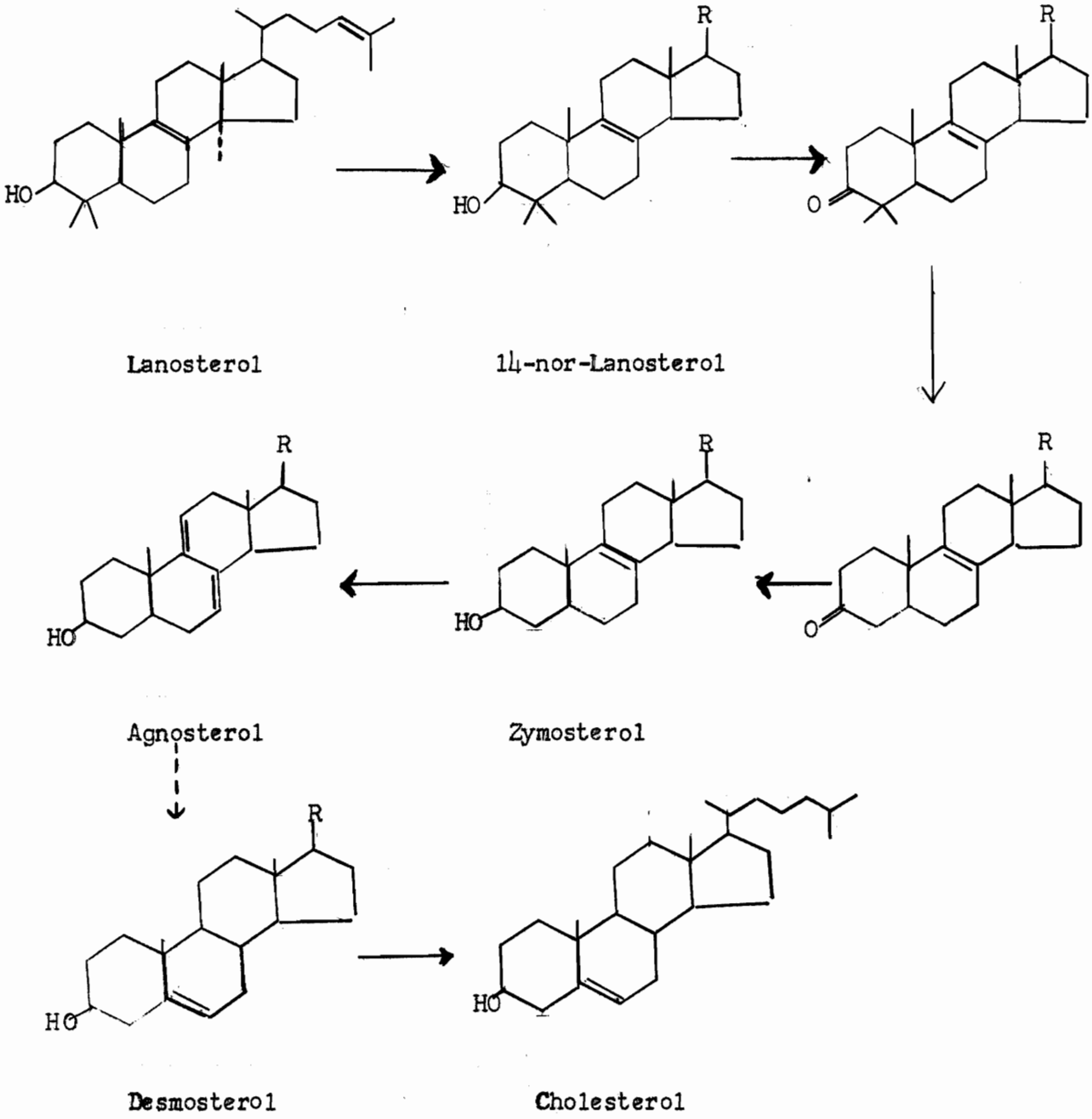


Figure 2. The conversion of lanosterol to cholesterol.

precursor of steroids depends upon measurements of the specific activity of cholesterol relative to that of some steroid compound concomitantly isolated from a biological system (124,186,187). It is now apparent that the intracellular distribution of cholesterol is such that, as in the case of squalene, only a fraction of that present in a steroid-forming cell is actually engaged in steroid biosynthesis. The existence of such "compartments" of unavailable cholesterol is responsible for the low specific activity of this compound in experiments in which the production of steroids from radioactive substrates is studied. Whereas the low specific activity of cholesterol compared with that of a simultaneously synthesized steroid appeared damaging to the acceptance of cholesterol as an obligatory intermediate in steroid synthesis, it is now clear that present data cannot exclude this possibility. In what follows it will be assumed that cholesterol is a precursor of all steroid hormones and the question of whether or not alternative pathways exist will be left open.

Cholesterol is subjected to side-chain cleavage with the formation of pregnenolone and isocaproic acid (188,189,253). All steroid-forming organs have been shown to be capable of converting pregnenolone to progesterone (190). In the testis progesterone is converted to androstenedione and testosterone (191). Progesterone loses its side-chain following 17-α-hydroxylation and 17-α-hydroxyprogesterone was established as an intermediate by Slaunwhite and Samuels who showed that testicular homogenate could convert progesterone-4-C^{14} to androstenedione and testosterone, but that as long as progesterone-4-C^{14} remained, 17-α-hydroxyprogesterone was always obtained

On the otherhand, 17-α-hydroxyprogesterone could give rise to the two androgens without detectable amounts of progesterone appearing.

Alternative Pathways.

(1) Forchielli and coworkers showed that ovarian tissue is capable of converting progesterone-7α-H^3 and 17α-hydroxyprogesterone-4-C^{14} to testosterone and Δ^4-androstene-3,17-dione in vitro (192). Moreover, the ratio of H^3 to C^{14} incorporated into these androgens was higher in the case of testosterone than androstenedione. This suggeste a pathway to testosterone which does not include 17α-hydroxy-progesterone or andostenedione as obligatory intermediates. This hypothesis was confirmed by incubating testis homogenate with progesterone-17α-H^3 as substrate. The resulting labeled testosterone was identified by its chromatographic behavior and constant specific activity in the presence of added carrier, by the behavior of the acetate and that of the hydrolyze acetate. Moreover, alkali caused progesterone-17α-H^3 to lose all its radioactivity while such treatment was without effect on the testosterone-17α-H^3. These facts provide striking evidence for a pathway from progesterone to testosterone which does not involve 17 α-hydroxypro-gesterone or androstenedione. The details of this pathway remain to be established.

(2) Kahnt and colleagues have produced evidence for a pathway from pregnenolone to DHEA as follows: (193,194)

Δ^5-pregnenolone $\longrightarrow$ 17 -hydroxy-Δ^5-pregnenolone $\longrightarrow$ DHEA.

The relative importance of this pathway and its possible role in the biosynthesis of testosterone remain to be determined.

B. Energy Requirements of Steroid Synthesis

The biosynthetic pathways thus briefly outlined are necessarily linked with reactions capable of fulfilling the large demands for energy which steroid biosynthesis involves. These requirements can be reduced to two fundamental sources of energy, namely, reduced TPN and ATP.

1. Reduced TPN. Reduced TPN is required for the following steps in the biosynthesis of testosterone.

(1) Two moles are required for the reduction of β-hydroxy-β-methylglutaryl CoA to mevalonic acid (twelve moles per mole of steroid) (195).

(2) One mole is required in the head to head fusion of two moles of farnesyl pyrophosphate to give squalene (196).

(3) One mole is required for the hydroxylation which initiates the folding of squalene (167,169).

(4) The splitting of the side-chain of cholesterol is a complex multistep process which requires at least one mole of reduced TPN (181,197,253).

(5) One mole is required for each of the three steps from progesterone to testosterone, i.e.:

(a) 17 hydroxylation (198)

(b) side-chain splitting (199)

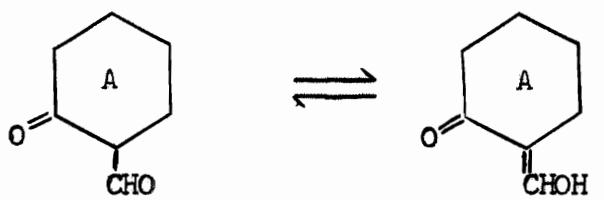
(c) 17β-hydroxysteroid dehydrogenation (200,201)

2. ATP. (1) Two moles of ATP are required to convert mevalonic acid in two steps to mevalonic pyrophosphate (171,176).

(2) One mole of ATP is required to convert mevalonic pyrophosphate to isopentenyl pyrophosphate (175,176) and 6 moles of the

latter are required for the production of one mole of steroid.

3. Miscellaneous. (1) The removal of three methyl groups from lanosterol involves oxidation and the mechanism is so far not certain, but may involve a hydroxymethylene intermediate since Pudles and Bloch have prepared such a compound (2-T, 4-C^{14}-4-hydroxymethylene-Δ^7-cholesten-3-one) and have demonstrated its conversion to tritium-containing cholesterol and $C^{14}O_2$ (202). The above compound exists in tantomeric equilibrium:



The reaction requires molecular oxygen and one mole of TPNH.

(2) It is generally held that the folding of squalene proceeds without special energy requirements beyond those of hydroxylation (252).

(3) The oxidation and reduction of the 3 hydroxyl group presumably returns the energy it consumes, while the reduction of the side-chain of desmosterol no doubt requires the energy equivalent of one mole of reduced TPN.

(4) The energy requirements of the double bond rearrangements between lanosterol and cholesterol are uncertain. Efforts to demonstrate a mechanism via a hydroxyl intermediate which undergoes dehydration, allowing rearrangement of the d ble bond, have so far failed and it is not possible to say whether such a rearrangement can proceed without net consumption of energy (203).

(5) One mole of reduced DPN is required (or preferred) for the 3β-hydroxysteroid dehydrogenase which converts pregnenolone to progesterone (204,205).

(6) Coenzyme A is used in the activation of acetate and acetoacetate and these reactions each require a mole of ATP.

It will therefore be seen that the production of one mole of testosterone from acetate requires at least

(1) 19 moles of reduced TPN

(2) 1 mole of reduced DPN

(3) 30 moles of ATP

in addition to the unknown requirements mentioned above. From this, it follows that whatever else stimulation of the production of testosterone may require, available TPN in the reduced form will be one important factor.

C. Sources of Energy for Steroid Synthesis.

1. Sources of reduced TPN - The important sources of TPNH are:

(1) Pentose phosphate shunt (206-210)

(2) Transhydrogenation of DPNH (211-215)

(3) Isocitric dehydrogenase in the Krebs cycle.

2. Fates of TPNH - The important fates of reduced TPN include: (206,207)

(1) Fatty acid synthesis (234-236)

(2) Steroid synthesis

(3) Amino acid synthesis (e.g., for the reductive amination of α-ketoglutarate to glutamate)

(4) Regeneration of the four carbon atom acids of the Krebs cycle, i.e., the reductive carboxylation of pyruvate to malate by the malic enzyme.

(5) Oxidation by a special respiratory chain.

It would therefore be logical to inquire whether steroid synthesis is specifically linked with the processes which produce reduced TPN or whether it enters into open competition with other routes of disposal of the reduced pyridine nucleotide. At present no final answer can be given to this question, but it would seem that TPNH could be provided in six possible ways:

(1) Specific stimulation of the pentose phosphate pathway, which provides two moles of the reduced TPN for every mole of glucose-6-PO_4 which enters the pathway (206-210).

(2) Inhibition of competitive processes which also use TPNH e.g., fatty acid or amino acid synthesis (206-210,234-236).

(3) Stimulation of transhydrogenation (211-215).

(4) Synthesis de novo of TPN.

(5) Transphosphorylation of DPN to TPN (216).

(6) Geometrical arrangement of subcellular compartments within which steroid synthesis can proceed in an environment free from competitive processes also requiring TPNH (206,207,217,218).

In general, it seems likely that synthesis de novo would not meet the immediate demands for reduced TPN and in any case the reduction of newly synthesized pyridine nucleotide will still be required. At present there are insufficient data to establish the existence of a system of compartments within the cell set aside for steroid synthesis. Among the remaining four alternatives there is

little positive evidence on which to decide. The concept of specific stimulation of the pentose phosphate pathway has received most attention. There is evidence for enhanced phosphorylase activity during stimulation of steroid synthesis (137-140) and the resulting increase in glycogenolysis is believed to promote increased disposal of glucose-6-PO_4 by way of the pentose phosphate pathway (219-229). It is too early to comment on transhydrogenation and transphosphorylation in relation to the needs of steroid synthesis.

At present a number of serious difficulties remain to be solved before it is possible to see these problems in perspective. In addition to the problem of the relative disposal of glucose-6-PO_4 by way of the pentose phosphate pathway compared with glycolysis, there remains the uncertainty about the state of oxidation of pyridine nucleotides within living cells and the difficulty of concluding that the behavior of added pyridine nucleotide adequately reflects the state of endogenous TPN and DPN. These problems are related to the consideration of intracellular compartments. Present evidence suggests that TPN within cells is largely present in reduced form and occupies the role of directing reductive synthetic activity, while DPN is chiefly present in oxidized form and plays an important part in metabolic oxidations (206,207). It is further believed that oxidation of DPNH is very much more active than that of TPNH and that this state of differential oxidation, so important to the smooth function of the two principal activities of living cells, namely the production of energy on the one hand and synthetic activity on the other hand, is maintained by a system of geometrical isolation within the cell which allows the simultaneous existence of "reduced" areas

and of "oxidized" areas (206,207,217,218). However, the evidence for this hypothesis is indirect and the extent, the boundaries and the contents of such intracellular compartments are quite unknown.

It, therefore, remains to be shown how the large requirements for TPNH during steroid synthesis are met by steroid-forming cells and whether the mechanism(s) involved is (are) controlled by a coupled or feedback system automatically adjusting production to current needs or whether a specific stimulating influence, such as a trophic hormone, is needed to provide the required TPNH for the increased steroid production in response to physiological stimulation.

3. Sources of ATP - Less attention has been given to the provision of ATP which no doubt comes from oxidative phosphorylation and it has been suggested that in this process the availability of ADP may be a limiting factor, with the result that the rate of utilization of ATP will govern the rate of oxidative phosphorylation by providing the ADP and inorganic phosphate resulting from its utilization (251).

D. The Rate of Steroid Synthesis.

Although the regulation of the rate of steroid synthesis is incompletely understood, there is evidence that the rate of biosynthesis of cholesterol is governed by the rate of one slow step which is rate-limiting. Bucher et al. showed that numerous factors which alter the rate of sterol synthesis (e.g., fasting, cholesterol feeding, triton and X-irradiation), exert their influence upon a reaction catalyzed by a microsomal enzyme which precedes the synthesis of mevalonic acid (230). Their data clearly establish that this

rate-limiting step is that between β-hydroxy-β-methylglutaryl CoA and mevalonic acid. The work of Gould and Popják (231) and that of Scarfe and Migicovsky (232) confirms the findings of Bucher *et al.* (230) and it appears that dietary cholesterol regulates the rate of sterol biosynthesis by suppressing the same reaction; this suggests that the regulation of sterol synthesis may be controlled by a feedback mechanism.

During the conversion of cholesterol to steroid hormones it is likely, from the work of Hechter and colleagues (132) (see page 33), that a rate-limiting step occurs which is sensitive to the influence of ACTH in the case of the adrenal cortex; the reaction involved is the conversion of cholesterol to pregnenolone.

Since it is probable that the cholesterol within a cell is compartmentalized so that only a portion of the whole is involved in steroid synthesis, it cannot be said with certainty that factors which apply to the rate of synthesis of cholesterol in general, necessarily apply (or apply with equal force) to the fraction which is engaged in steroid synthesis.

III. THE BIOSYNTHESIS OF FATTY ACIDS

In that the biosynthesis of fatty acids shares with that of steroid hormones the need for reduced TPN, it is necessary to consider the possibility that these two activities may compete for the available supply of reduced pyridine nucleotide.

At present the detailed mechanism of fatty acid biosynthesis is not understood. Vagelos and coworkers have isolated a bacterial enzyme capable of synthesizing palmitic acid in the presence of malonyl CoA, acetyl CoA, TPNH and carbon dioxide (234). Following an initial condensation reaction involving these substances, the fatty acid molecule is built up by recurring additions of a carbon atom fragment to the product of condensation. Present interest centers around the nature of the product of condensation (234-236, 244,245). Vagelos and Alberts favor a concerted condensation and decarboxylation reaction between malonyl CoA and acetyl CoA (234); this reaction is catalyzed by an enzyme which these workers have purified. The postulated condensation product is a β-keto-acyl CoA derivative and the authors believe that the one-step reaction is more likely to overcome the unfavorable energy levels involved in alternative two-step mechanisms (234). The same group has purified a long chain fatty acid synthesizing enzyme (244) and have studied the synthesis of branched chain and odd-numbered fatty

acids (245); their findings indicate that the synthesis of one mole of palmitate in their system, requires approximately 14.7 moles of TPNH (224).

The epididymal fat pad has been used extensively for the study of fatty acid synthesis (244,245) and several workers have been able to demonstrate a striking release of non-esterified fatty acids from the cells of this tissue under the influence of ACTH (237-243). Unfortunately the role of ACTH in the control of fatty acid synthesis is much less clearly defined. The hormone exerts a ketogenic and adipokinetic action in adrenalectomized rats and mice (237-239). Engel and colleagues have shown that these effects result from a property inherent in the ACTH molecule and are not due to the influence of contaminants (239).

The Problem - The literature reviewed in this chapter would seem to show that the time is ready for a study of the mechanism of action of gonadotrophic hormones. Effects of these hormones in vitro have been described, the hormones are available in a relatively pure state, the biosynthetic pathway of steroid hormones is now well established and important contributions have already been made to the mechanism of action of corticotrophin, which shares with ICSH the capacity to stimulate the production of steroid hormones. The concept of a mechanism common to both ICSH and ACTH by which the stimulation of steroid synthesis is effected, is esthetically appealing but without experimental foundation. Although the interpretation of present findings concerning the mechanism of action of corticotrophin may be open to debate, these findings are reproducible and significant. One approach to the search for the mechanism of action

of ICSH would be guided by analogy with studies of ACTH; it would be important to establish differences or similarities.

The mechanism by which any physiological process takes place is obviously important, not only because of its intrinsic academic interest, but because it may point the way to a general principle - to a mechanism used by similar systems. Moreover, the understanding of mechanism is the understanding of control and control or loss of control lies at the heart of many biological mysteries, both physiological and morbid.

Since Brady has demonstrated an effect in vitro of chorionic gonadotrophin upon steroid synthesis, it would now be logical to see whether ICSH, which shares with chorionic gonadotrophin its most striking physiological effect, namely, Leydig cell stimulation, is also capable of showing the same action in vitro. If this turns out to be the case, it may prove possible to measure, albeit crudely, the response of Leydig cells to ICSH and to use such measurements as a gross index of the influence of factors which alter the in vitro production of steroids by the adrenal cortex.

The problem then, is to approach experimentally the mechanism of action of ICSH and since the response to this hormone is chemical, the experiments will need to be conceived at a molecular level. Although present methods may allow no more than a preliminary exploration of this complex project, it may be hoped that such an exploration will prove of some value.

The succeeding chapters deal with experiments with the following general goals:

(1) To find a reproducible and measurable response to ICSH *in vitro*.

(2) To study possible synergism between the two gonadotrophins ICSH and FSH.

(3) To use such findings to test the effect of factors which might be relevant to the mechanism of action of ICSH.

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CHAPTER II

MATERIAL AND METHODS

1. Experimental Animals

   A. Rabbits

   B. Dogs

A. Rabbits - Since the animal in which Brady reported stimulation of testicular slices by HCG was the rabbit(21), it was decided to use adult male rabbits between one and three years of age in the present studies. The rabbit was also suitable because, apart from its size and general ease of handling, the principal testicular androgen of this species is testosterone and testosterone is conveniently identified by acetylation and oxidation.

The rabbits used were of the white New Zealand strain but an absolutely homogeneous strain was not available. The animals were isolated from females for at least two weeks before the experiments and were kept in individual cages. The rabbits weighed between 3.5 and 4.5 kg at the time of sacrifice.

Pretreatment. Hormones were administered either subcutaneously through an 18 gauge needle or intravenously through a 22 gauge needle. Human chorionic gonadotrophin, follicle-stimulating hormone and interstitial cell-stimulating hormone were dissolved in demineralized water shortly before administration. Testosterone was administered

as an oily solution of the propionate. When HCG was used to produce prolonged stimulation of Leydig cells, it was given subcutaneously on alternate days for three injections, the last of which was administered two days before the experiment, according to the method used by Mason in the case of dogs (1).

B. Dogs - Adult male mongrel dogs were used. The animals were obtained from the city pound and were kept in the department animal house for several days before each experiment.

2. Preparation of Tissue

A. Testis. The testes were removed from rabbits which had been rendered unconscious by a blow on the head, and were dropped into ice-cold Krebs-Ringer bicarbonate buffer. When animals were to be kept alive following unilateral castration, intravenous Nembutal (30 mg per kg body weight) was administered slowly, as the anaesthetic.

I. Slices - The testes were prepared for slicing by removing adherent fat, tunica albuginea, epididymis and epididymal fat pad. The tunica intima was peeled off and the organ cut into four pieces by means of scissors. The pieces were placed in ice-cold buffer and sliced with the Stadie-Riggs slicer (2). A new blade was used for each experiment and before use was soaked successively in boiling ethanol, ether and two saline baths. Slices were easily prepared by using short to and fro sawing movements with the blade, instead of one steady continuous sweeping action. The instrument yields slices approximately 0.5 mm in thickness (2). The slices were kept in ice-cold buffer and weighed on a watchglass after gentle drying with filter paper. The weighed slices were then placed in the incubation flask.

II. Homogenate - Testis homogenate was prepared in a 20 ml Ten-Broeck glass homogenizer from pieces of testis, cut from the whole organ, as in the case of slices. An appropriate volume of buffer was placed in the homogenizer, the pieces of testis added and homogenization continued until a uniform liquid preparation was obtained and the surfaces of plunger and barrel were felt to engage. This process was interrupted from time to time to place the homogenizer and its contents in crushed ice for periods of a half to one minute.

When whole homogenate was used, the homogenized testis was made to a convenient volume with cold buffer. When it was desired to remove cell debris and nuclei, the homogenate was centrifuged in a 10 ml plastic cup at 0°C for 15 minutes at 650 x g. The supernatant layer was decanted into a cylindrical graduate and buffer added to a convenient volume.

B. Pituitary. Dogs were anesthetized by intravenous Nembutal, 25 mg per kg body weight and a circular flap of bone approximately 6 cm in diameter was removed from the skull by means of a bone saw. The cerebral hemispheres were lifted from the base of the skull to expose the pituitary stalk, which was severed and the gland removed from the sella turcica while the animal was still alive. The pituitary was placed in a test tube surrounded by ice and the anterior pituitary was separated from the posterior pituitary by means of forceps before the next step.

(i) Slices. Slices of pituitary tissue were prepared by fine scissors and weighed on a watchglass. The canine pituitary gland proved unsuitable for the use of a Stadie-Riggs slicer or a razor blade.

(ii) Homogenate. A measured volume of cold buffer was placed in a Ten-Broeck glass tissue-grinder of 10 ml capacity and the whole anterior pituitary added. The tissue was readily homogenized to a uniform pink liquid by a few passes of the plunger. The whole homogenate was made to a convenient volume with buffer and used without centrifugation.

C. Hypothalamus - The dog was prepared as before and the whole brain removed with the pituitary attached. The brain was divided in the saggital plane by cutting the corpus collosum, and a block of tissue including the whole hypothalamus, removed from each half. One block was prepared for microscopic study to identify the structures dissected in the other half. The latter was dissected with a sharp, thin brain knife and the required nuclei removed and placed in a test tube surrounded by ice.

Slices of hypothalamic tissue were prepared by means of fine scissors; this method provided more uniform slices than the instrument described by Magno and Bunker (3). Slices were weighed on a watch glass. In these experiments pituitary tissue was removed by Dr. K. Eik-Nes and hypothalamic tissue was prepared by Dr. K. R. Brizzee of the University of Utah.

D. Miscellaneous. Adrenal glands were removed through a midline abdominal incision, the animal being anesthetized by intravenous Nembutal (30 mg per kg body weight). Slices of adrenal tissue were prepared by means of fine scissors.

Kidneys were removed through a similar incision under the same anesthetic and slices prepared by the Stadie-Riggs slicer.

3. Buffers

Slices of testis were incubated in Krebs-Ringer bicarbonate buffer (4), which was prepared one hour before each experiment from stock solutions stored at 18°C. Stock solutions were replaced every four weeks and the freshly diluted buffer was not kept. The newly prepared buffer was briskly gassed with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide until quite clear (approximately 10 minute and was kept ice-cold thereafter until the incubation began.

Homogenates of testis were prepared in 0.1M phosphate buffer (42), enriched with salts, glucose and cofactors as described by Bucher et al. (29), as follows:

Magnesium Chloride	0.004M
Versene	0.001M
Nicotinamide	0.03 M
Glucose	0.125M
Cysteine Hydrochloride	0.01 M

The pH was adjuseted to 7.4 by addition of the appropriate quantity of molar K_2HPO_4 or KH_2PO_4 (42).

Pituitary tissue was incubated in Krebs-Ringer bicarbonate buffer enriched with succinate 7.8×10^{-3}M (5,6,7).

4. Substrates.

A. Radioactive Substrates - Table VII indicates the nature and source of the radioactive substances used. Water soluble substrates were added to the incubation flasks from stock solutions which were kept frozen between experiments. Cholesterol and steroid substrates were stored in vacuum redistilled methanol and were dried under nitrogen in 15 ml conical centrifuge tubes before use. The dry material

was dissolved in 0.3 ml propylene glycol; this solution was added to the incubation flask by pouring the buffer from the flask onto the propylene glycol and washing the mixture to and fro several times between the two vessels and finally draining the centrifuge tube into the flask.

Radioactive substrates were diluted with unlabeled material when necessary. The radiochemical purity of these substances was indicated by chromatographic evidence provided by the manufacturers.

Table VII

Substance	Source	Lot No.	Specific activity
Sodium Acetate-1-C^{14}	New England Nuclear	65-19-4	20 mc per mMole
Sodium Mevalonate-2-C^{14}	" " "	16-19-2	2.7 mc per mMole
Cholesterol-4-C^{14}	" " "	64-68B-4	2.73 mc per mMole
Testosterone-4-C^{14}	Tracerlab Co.	376-139A-2	3.7 mc per mMole
Δ^5-Pregnenolone-7-H^3	" "	16-24-25	0.7 mc per mMole
Sodium Butyrate-1-C^{14}	" "	67-23-5	1.96 mc per mMole
α-Aminoisobutyric Acid-1-C^{14}	" "	46-132-1-3	2.9 mc per mMole
Progesterone-21-C^{14}	" "	18-21-23	2.4 mc per mMole
D-Xylose-1-C^{14}	Nat. Bur. Std's.	-	0.7 mc per mMole
DL-Tryptophan-1'-C^{14} (2-C^{14}-alanine)	California Corp.	106758	7.65 mc per mMole
L-Valine-1-C^{14}	" "	108945	12 mc per mMole

B. Miscellaneous. Table VIII shows the remaining substances used as substrates or test substances.

Table VIII

Substance	Source	Grade of Purity
Sodium Acetate Anhydrous	Mallinckrodt	AR
Glucose	Mallinckrodt	AR
Sodium Succinate	Matheson Co., Inc.	AR
Cholesterol	National Biochemicals Corp.	AR
Testosterone	Dextran Chemicals Inc.	USP
Androstenedione	Dextran Chemicals Inc.	USP
Testosterone Acetate	Dextran Chemicals Inc.	USP
Sucrose	Mallinckrodt	USP
Nicotinamide	Mann Research Laboratories	USP 98.5%min
Versene	Eastman Organic Chemicals	-
L-Cysteine Hydrochloride Monohydrate	Mann Research Laboratories	CP
Chloramphemicol	Pfizer	-
Puromycin	American Cyanamid Company	-
PPO (2,5-diphenyl-oxazole	Packard Company	Scintillatic grade
POPOP (1,4-bis-2-(5-phenylox-azolyl)-benzene	Packard Company	Scintillatic grade
Chromium trioxide	Baker and Adamson (stored in a desiccator)	AR
Acetic anhydride	J. T. Baker Chemical Co. (stored in a desiccator)	AR
Acetic acid	Baker and Adamson (stored in a desiccator)	AR
Phenol Reagent	Medical Chemical Corporation	-
Pyridine	J. T. Baker and Company (stored in a desiccator)	AR
Formic Acid	Matheson, Coleman and Bell	98-100%

Substance	Source	Grade of Purity
Serum Albumin (Bovine)	Prepared by Dr. S. R. Dickman, University of Utah (Stored in a desiccator)	Recrystallized four times
Plasma Protein (Human Fraction III)	University Laboratory of Physical Chemistry, Harvard University	

C. Solvents. Table IX indicates the nature and source of organic solvents used.

Table IX

Solvent	Source	Purity
Ether	Mallinckrodt	AR
Chloroform	Mallinckrodt	AR
Acetone	Mallinckrodt	AR
Benzene°	Mallinckrodt	AR
Methanol[x]	Mallinckrodt	AR
Pentane[+]	Skellysolve Company	-
Hexane[+]	Skellysolve Company	-
Propylene Glycol	Eastman Organic Chemicals	USP
Methyl Cyclohexane	Eastman Organic Chemicals	-
Ligroin[+]	Skellysolve Company	-
Formamide	Fisher Scientific Chemical	AR
Toluene°	Mallinckrodt	AR
Ethyl Acetate[+]	Mallinckrodt	AR

° Redistilled over sodium before use

x Vacuum redistilled before use

+ Redistilled before use

5. Cofactors. Table X shows the nature and source of the cofactors used.

Table X

Cofactor	Source	Lot No.	Presentation
Triphosopyridine Nucleotide monosodium	Sigma (from yeast DPN)	11B-745	Powder, Assay 99%
β-Diphosphopyridine Nucleotide	Sigma (from yeast)	41B-679-10	Powder, Assay 99%
Glucose-6-Phosphate Barium salt	Sigma	110B-26-1	Crystalline, Assay 98-100%
Glucose-6-Phosphate Dehydrogenase (Type V)	Sigma	120B-683	Powder, 90 K units/mg protein
Adenosine-3',5'-cyclic Phosphoric acid	Sigma	79-651-2	Crystalline, Assay 98-100%

6. Protein and Peptide Hormones

I. Follicle-Stimulating Hormone. Two preparations of FSH were used in these experiments. A. NIH-FSH-S1 kindly provided by Dr. A. E. Wilhelmi. B. A preparation made available by Dr. S. L. Steelman, hereafter called FSH(Steelman).

A. NIH-FSH-S1. This material was presented in two ways - as a powder and as a sterile-filtered, lyophilized powder in a vial. The former preparation, which was not sterilized, is referred to as FSH (powder form), the second will be called FSH (vial form). Both were readily soluble in water and in Kreb's-Ringer bicarbonate buffer.

Preparation. This substance was prepared from sheep pituitary according to the method of Ellis (8).

Potency. The potency of this preparation was measured by two methods:

(i) Steelman - Pohley HCG augmentation method (19).

(ii) Ovarian weight method.

(i) Steelman-Pohley Method (19). Immature Sprague-Dawley rats (26 days old) were injected three times each day for three days according to the scheme proposed by Steelman and Pohley and autopsy was performed on the fourth day. The augmenting dose of HCG was 20 I.U. The results of this assay are shown in Table XI.

Table XI

Assay of NIH-FSH-S1 by Steelman-Pohley Method

Date	Potency compared with Armour Standard #264-151-X
1. March 7, 1958	2.8
2. March 17, 1958	2.7
3. October 9, 1958	2.6

Assays 1 and 2 were performed on the powder form and assay 3 on the vial form (i.e., on part of the powder form which was sterilized between the times of assays 2 and 3).

(ii) Ovarian Weight Method. The procedure was the same as that outlined in (i), except that no HCG was used and FSH was injected once daily for 4 days into 25 day old Sprague-Dawley rats; autopsy was performed on the fifth day. The dose required to double ovarian weight in this test was 250 μg.

Contamination. Table XII shows the results of assay for other protein hormones.

Table XII

Examination of NIH-FSH-S1 for contaminating protein hormones

Hormone	Amount Found	Method
TSH	< 0.01 USP units/mg protein	P^{32} uptake by chick thyroid (9)
ACTH	0.44 milliunits/mg protein	Ascorbic acid depletion (10)
Growth Hormone	Absent from 2.5 mg doses of FSH	Ten day weight gain test (11)
Prolactin	(a) < 0.01 units/mg protein (b) < 0.15 units/mg protein	Crop gland test (12) (a) local injection (b) systemic injection
ICSH	(In terms of NIH-LH 227-80) 0.006 mg/mg protein	Ovarian Ascorbic Acid assay of Parlow (13)

B. FSH (Steelman). This material was prepared by the method described by Steelman et al. (20). In the Steelman-Pohley assay (19), this FSH showed a potency 2-3 times that of the Armour Standard FSH (264-151x) (43). However, the material was found to contain considerable ICSH, which in the ascorbic acid depletion test (13) was shown to have a potency of 0.05 that of Armour Standard ICSH (227-80) (43). This FSH preparation showed less than 0.1 USP units per mg of TSH in the P^{32} uptake test (9).

II. Interstitial Cell-stimulating Hormone. Three preparations of ICSH were used:

A. NIH-LH-S-1 (Ovine preparation).

B. A preparation kindly given by Dr. C. H. Li; this was an ovine preparation, hereafter called ICSH (Berkeley).

C. A preparation provided by Dr. Darrell N. Ward; this was an ovine preparation, hereafter called ICSH (Houston).

A. NIH-LH-S-1.

Preparation. This hormone was prepared from sheep pituitary after the method of Ellis (8).

Potency. This preparation was measured for ICSH activity against Armour LH Standard (227-80) by two methods:

(i) Ventral Prostate Method (14)

(ii) Ovarian Hyperemia Method (15)

(i) Ventral Prostate Method (14). Measurement of weight increase in the ventral prostate of hypophysectomized rats indicated that the present preparation is equal in potency to the Armour Standard (227-80).

(ii) Ovarian Hyperemia Method (15). Intravenous injection into immature rats showed that 0.4 μg was the minimal dose required to produce hyperemia, while 1.2 μg gave a maximal response. The present preparation is equal in potency to the Armour Standard (227-80) in this assay.

Contamination. Table XIII shows the results of assay for other protein hormones.

Table XIII

Examination of NIH-LH-S-1 for contaminating protein hormones

Hormone	Amount Found	Method
TSH	0.015 USP units/mg protein	P^{32} uptake by chick thyroid (9)
ACTH	0.25 USP milliunits/mg protein	Ascorbic acid depletion (10)
Growth Hormone	Absent from 1.0 mg doses of LH	Ten day weight gain test (11)
Prolactin	Absent from 25 mg doses of LH	Pigeon crop gland tests (12)
FSH	Absent from 6.0 mg doses of LH	Ovarian weight test (see page 94)

B. ICSH (Berkeley). Dr. C. H. Li prepared sheep ICSH according to the method described by Squire and Li (16). The criteria of purity of this preparation are physico-chemical (16).

C. ICSH (Houston). Dr. Ward kindly provided a sample of his sheep LH_2 (4-177D). This material was prepared by the Koenig-King ethanol-acetate extraction and chromatography on carboxymethyl cellulose. The method has been reported in detail (17) and the final product was assayed by two methods:

(i) Ovarian ascorbic acid depletion (13) revealed a potency of 1.93 relative to Armour Standard LH-227-80.

(ii) Ventral prostate weight increase (14) in hypophysectomized rats indicated a potency of 1.98 relative to the same standard. These results were calculated by the two-dose factorial assay of Bliss (18). Physicochemical evidence of purity has been given for this material but details of bioassays for contaminating hormones have not been published.

III. Miscellaneous. Table XIV shows the source and nature of the remaining protein and peptide compounds used.

Table XIV

Source and Nature of Protein and Peptide Compounds

Hormone	Source	Presentation	Assay
Human Chorionic Gonadotrophin	(i) Ayerst Lab. (ii) Upjohn Co.	Solution in 0.8% NaCl. 1000 I.U./ml. Powder	Ovarian weight† (Rat)
Corticotrophin	Upjohn Co.	Powder	Ascorbic acid Depletion (10)
Thyrotrophic hormone	Armour Lab.	Powder	P^{32} uptake (9)
Growth Hormone (Bovine)	Armour Lab.	Powder	Ten day weight gain test (11)
L_8-Vasopressin	Sandoz	Solution in 0.8% NaCl 24 units/ml	Pressor effect in the rat (44)
Melanocyte-stimulating hormone	Dr. S. L. Steelman	Powder	Frog skin assay (45)

† The British Pharmacopoeia, 1948. pp. 804-806.

7. Incubation. Incubation was performed in a Dubnoff metabolic incubator at 37.5°C for 1 to 6 hours, with constant agitation. The incubation chamber was continuously gassed with 95 per cent oxygen and 5 per cent carbon dioxide throughout the incubation. The tissue and medium were placed in open Erlenmeyer flasks (25 ml or 50 ml) and the contents were added in the order shown on page 117. The tissue was always added last and the expression "final volume" refers to the volume of the medium to which slices of tissue were added but includes the volume of tissue in the case of homogenates. Unless otherwise stated, the final volume was 3.0 ml in a 25 ml Erlenmeyer flask.

8. Extraction. At the conclusion of incubation the contents of each flask were transferred to a Ten-Broeck glass homogenizer (10 ml capacity) and the slices were homogenized to a uniform gray liquid; this step was omitted in the case of testicular homogenates. A sample (0.1 ml) of the homogenized tissue was removed for protein estimation and the remainder was transferred to an 80 ml centrifuge tube in which 100 μg of unlabeled testosterone dissolved in 0.1 ml of methanol had already been placed. The incubation flask was then rinsed with 4 ml of methanol which was added to the 80 ml centrifuge tube and was followed by 3 pellets of potassium hydroxide. These additions gave a suspension which was 66 per cent in methanol and 10 per cent in potassium hydroxide. The mixture was boiled for 10 minutes in a water bath and then allowed to cool. Five ml of distilled water was added to the cool solution.

This solution was extracted three times with ethyl ether (three volumes of ether to one of water phase). The first two extracts were combined and dried under nitrogen at 40°C; the third was added to the dry extract. The ether extract was then washed with 10 ml of 10 per cent aqueous sodium

acetate, followed by three washes with distilled water (10 ml each). These steps were performed with a footed stirring-rod and after each extraction and wash, the layer to be discarded was removed by means of a serum-lifter operated by compressed air. Each extraction and wash consisted of 200 passes with the footed stirring-rod.

Approximately 10 mg of anhydrous sodium sulfate was added to the extract to remove any water and the extract was transferred to a citrate tube by means of the serum-lifter and dried under nitrogen. The sides of this tube were washed six times with chloroform and the dry extract was dissolved in 20 ml of pentane and left in a refrigerator over night.

9. Column Chromatography. An aluminum oxide column was prepared according to Brady's modification (21) of the method of Samuels (22). Reagent grade aluminum oxide, obtained from Baker and Adamson (Code 1236) was used and this was activated at 600°C for 4 hours, being allowed to cool to 200°C before it was removed from the furnace. The aluminum oxide was then poured into 15 ml screw-cap vials and stored in a desiccator. Each batch of activated alumina was tested by applying a sample of testosterone-4-C^{14} dissolved in 20 ml of pentane to the column and eluting fractions as described below. The various fractions were dried under nitrogen in vials prepared for the Packard Tri-carb liquid scintillation counter and counted in this apparatus. If less than 90 per cent of the sample of testosterone-4-C^{14} was recovered in the expected fraction, the aluminum oxide was reactivated.

The column was prepared by pouring aluminum oxide into a glass column (without stop-cock), 29 cm long with an internal diameter of 1 cm. The column was packed dry by brisk tapping on the bench surface for two min-

utes, followed by tapping the sides of the column with a pencil and finally by tapping on the bench for a further two minutes. The aluminum oxide was retained in the column by a small piece of clean glass wool. The dry column was washed, the sample applied and elution carried out as follows:

Column wash	Pentane 15 ml
Sample applied in	Pentane 20 ml
Sample wash (Fraction A)	Pentane 15 ml
Fraction B	Pentane 15 ml
" C	Chloroform 3% in Pentane 30 ml
" D	" 6% " " 15 ml
" E	" 9% " " 30 ml
" F	" 12% " " 30 ml
" G	" 18% " " 30 ml
" X	Chloroform 20 ml

Cholesterol was removed in fractions C and D, while testosterone appeared in fraction G (21,22). The required fractions were dried under nitrogen.

10. Paper Chromatography. Samples were prepared for paper chromatography by drying under nitrogen and washing the sides of the containing vessel with chloroform six times, drying between each washing. The sample was dissolved in 6 drops of methanol-chloroform (1 to 1) and applied to paper strips by means of a thick walled Pasteur pipette. When the first solution had been applied, 4 drops of methanol-chloroform were added to the sample and the process of applying to paper was repeated; finally 2 drops of methanol-chloroform were added and this solution also

applied to paper.

Table XV shows the systems of paper chromatography used.

Table XV

System	Stationary Phase	Mobile Phase	Impregnation of paper	Duration
Zaffaroni (23,24)	Propylene Glycol	Ligroin	Propylene Glycol-Methanol 2:3	24 hrs
	Formamide	Hexane-Benzene 1:1	Formamide-Methanol 1:1	To the front
	Formamide	Hexane	"	"
Savard (25)	Methyl Cyclohexane	Ligroin	Methylcyclohexane-Methanol 2:3	24 hrs
Bush A (26)	Aqueous Methanol	Light Petroleum	-	To the front

For Zaffaroni and Savard systems, Whatman No. 1 filter paper for chromatography was used in strips 2 cm wide. For the Bush system Whatman No. 4 filter paper was used in strips 2 cm wide. Strips were dried over night before a fan; ultraviolet-absorbing areas were identified by means of a Haines' scanner (27) and marked with pencil. The strip was then ready to be counted for radioactivity.

Elution of paper chromatograms was performed with 5 ml of vacuum distilled methanol. The area to be eluted was cut from the paper strip with one end pointed. The paper was suspended from a curved 22 gauge needle attached to a 10 ml syringe. The methanol was placed in the barrel of the syringe which was clamped in position so that the methanol ran down the needle, over the paper and slowly dripped into a receiving vessel.

11. Measurement of Radioactivity.

(a) Strip Counting. Dry chromatograms were counted in a window Geiger counter and radioactivity was recorded on a Brown Electronik Recorder, made by Minneapolis Honeywell Regulator Company. The counter was calibrated against a windowless plate counter by applying solutions of progesterone-21-C^{14} to a paper chromatogram developed in hexane-formamide. Identical aliquots were plated on aluminum planchettes and counted in a plate-counter. Various amounts of progesterone-21-C^{14} were used to calibrate the instrument over a wide range of radioactivity. The area under the peak representing progesterone-21-C^{14} was measured by triangulation and through the range tested, one square centimeter on the 100 Scale of the recorder, represented approximately 22 counts per minute on the windowless plate counter.

Chromatograms were examined by means of the strip counter and the areas of peaks representing radioactivity were measured by triangulation and converted to counts per minute (CPM) by means of the factor 22. The figures resulting from this conversion were more convenient than those expressed in square centimeters and in each experiment comparison of one specimen with another was based on CPM. No attempt was made to achieve absolute values by conversion to DPM.

(b) Liquid Scintillation Counting. Samples were counted in the Packard Tri-Carb Liquid Scintillation Spectrometer Model 314X, by adding 10 ml of scintillation fluid. Scintillation fluid was prepared by dissolving 4 gm of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2 (5-phenyloxazolyl)-benzene in one liter of toluene redistilled over sodium; this fluid was stored in a brown bottle.

When aqueous solutions were counted, 0.2 ml aliquots were mixed with 3 ml of absolute ethanol and 7 ml of scintillation fluid were added. Protein precipitates and samples of tissue were ground with a mortar and pestle, suspended in 3 ml of ethanol and 7 ml of a gel prepared by making a 4 per cent solution of cab-o-sil in scintillation fluid.

Paper chromatograms were counted by cutting the strip into pieces 1 cm by 2 cm. These were placed in counting vials and 10 ml of scintillation fluid added. Equal areas of paper above and below that occupied by the sample were similarly counted to provide control levels.

(c) Plate counting. A Nuclear Chicago windowless plate counter was used to determine the specific activity of protein precipitates. The precipitate was dissolved in formic acid by dropwise addition and 0.2 ml aliquots of this solution were delivered to the center of a weighed aluminum planchette. The formic acid solution spread evenly over the planchette and dried to constant weight after remaining for two hours in a hood.

12. Identification of Testosterone. The radioactive material which was extracted following incubation and which accompanied unlabeled carrier testosterone was identified as testosterone by the following procedures:

A. In all experiments the radioactive peak exactly coincided with the area occupied by the added carrier testosterone.

B. Acetylation. The area of paper chromatogram occupied by carrier testosterone was eluted; the eluate was dried under nitrogen and acetylated. Acetylation was performed by adding 0.4 ml of a mixture of pyridine and acetic anhydride (5:1) to the dried eluate. The mixture was shaken and left for 24 hours at room temperature in the dark.

At the end of this time the reaction mixture was dried under nitrogen as described by Fevold (28). The acetylated compound was subjected to paper chromatography in the Zaffaroni system hexane-benzene-formamide.

The dry strip was examined by means of a Haines' scanner (27) and counted in a strip counter. The ultraviolet-absorbing testosterone acetate formed from carrier testosterone was accompanied by a single peak of radioactivity and both showed an R_f value of 0.82, as expected for testosterone acetate in this system.

C. Oxidation. Material eluted as in B, was oxidized by chromic acid. Crystalline chromic acid was repeatedly washed with glacial acetic acid to remove moisture and 0.4 ml of the final solution of chromic acid in glacial acetic acid, was added to the dried eluate and allowed to stand for 12 hours. At the end of this time 5 ml of water was added and the solution extracted three times by shaking with 20 ml of ether. The combined extracts were dried under nitrogen and applied to a paper chromatogram in the Zaffaroni system hexane-benzene-formamide. The oxidized carrier was accompanied by a single peak of radioactivity showing an R_f value of 0.6, consistent with the behavior of androstenedione.

D. Recrystallization. Recrystallization of radioactive products and their derivatives was performed to constant specific activity. This procedure was carried out in a test tube (8.0 x 75 mm). The sample was transferred in chloroform to the test tube by means of a Pasteur pipette and dried under nitrogen. An appropriate weight of unlabeled carrier was added in crystalline form and the mixture left in a desiccator for 24 hours before weighing. The mixture was then dissolved in a suitable volume of the selected solvent by warming in a water bath.

An aliquot of the solution was removed by means of a micropipette and transferred to a counting vial, dried and stored in a desiccator. Crystallization was allowed to begin at room temperature and with the appearance of the first crystals the stoppered test tube was transferred to the refrigerator. When crystallization was complete, the mother liquor was removed with a Pasteur pipette, the crystals dried under nitrogen and left in a desiccator for 24 hours before weighing. The crystals were again dissolved in a suitable volume of the next solvent, an aliquot removed and the process repeated. Scintillation fluid (10 ml) was added to each of the dried aliquots, which were then counted in the liquid scintillation counter.

E. Gas Chromatography. The retention time of radioactive material isolated from incubated tissue was measured by gas chromatography. The material tested was pooled from six flasks and extracted without addition of carrier testosterone. The specimens were run in the system ligroin-propylene-glycol as usual and the radioactive peaks which moved the same distance as carrier testosterone on a parallel strip were eluted, pooled, dried under nitrogen and studied by gas chromatography.

13. Extraction of Fatty Acids. When analysis of the fatty acid content of tissues was undertaken, the water phase after extraction with ether was acidified to pH 1 by means of 12 N hydrochloric acid with the aid of pHydrion paper and extracted three times with three volumes of petroleum ether. The first two petroleum ether extracts were dried under nitrogen and the third added to the dry extract. The petroleum ether solution was washed once with 10 ml of 1 M acetic acid and four times (10 ml each) with water, which caused the pH to return to 6.

The petroleum ether phase was dried under nitrogen until no smell of acetic acid could be detected. The last step was performed in a counting vial or in a test tube according to the particular experiment. When performed in a test tube, this was sealed under nitrogen before being sent for gas chromatographic studies.

14. Gas Chromatography (30-40). Gas chromatographic analyses were performed on a Wheelco model 10 "Gas-liquid Chromatograph". This instrument was made available through the courtesy of Dr. E. C. Horning at the National Institutes of Health, Bethesda, Maryland.

For the study of fatty acids, the liquid phase was ethylene glycol-adipate and the column was packed by solution in chloroform and vibration (30) in a U-tube 4 mm in internal diameter and 2 meters long.

Steroid compounds were studied on a Barber-Colman gas chromatograph by Dr. E. Nishizawa and Dr. K. Eik-Nes. The liquid phase consisted of neopentenylglycolsuccinate polyester. Again the column was packed by a solution technique (30) using a 10 per cent solution of the polyester in chloroform and 60-80 mesh acid-washed "Celite 545". This coated celite contained 20 per cent (w/w) of stationary phase and was packed by vibration in a glass U-column, 100 cm by 2 mm internal diameter. The whole column was conditioned at 200°C and the gas phase was argon at a pressure of 18 pounds per square inch. A potential of 750 volts was used in the ionization detector.

15. Permeability Studies. The compounds D-xylose-1-C^{14} and α-amino-isobutyric acid-1-C^{14} were used to study the influence of hormones on cell permeability. Aqueous solutions of these substances were added to the incubation medium and at the conclusion of the incubation the slices were carefully transferred to a thick walled, pyrex ignition tube (125 x

16 mm) and 10 ml of Krebs-Ringer bicarbonate buffer added. The contents of the tube were gently mixed by inversion (the top of the tube being sealed with Parafilm) and centrifuged at 0°C until the centrifuge reached 1,500 x g; the centrifuge was then switched off and allowed to come to a stop. Five such washes were performed and from the conclusion of the incubation the slices were kept at 0°C.

The slices were drained and then dried in an oven at 120° for 2 hours, cooled, weighed and returned to the oven for a further hour. This process was repeated until a constant weight was obtained. The tissue was then brought to a boil in 3 ml of water in stoppered centrifuge tubes, by means of a boiling water bath. The tissue was agitated with a stirring rod and left to cool; an aliquot of the cool solution was counted in the liquid scintillation counter. Aliquots of the incubation medium and of the combined buffer washings and a suspension of the tissue remaining after boiling were also counted in the liquid scintillation counter.

16. Amino Acid Incorporation Studies. C^{14}-labeled amino acids were added to the incubation flasks and incubation conducted in the usual way. At the end of incubation, the slices were homogenized and centrifuged at 650 x g for 15 minutes. The supernatant was decanted in 0.4 ml of 40 per cent aqueous trichloracetic acid, extracted with a stirring rod (100 strokes), boiled for two minutes and extracted again. The mixture was centrifuged at 650 x g for two minutes and the supernatant discarded. This process was repeated twice, using 4 ml of 4 per cent trichloracetic acid. Two extractions with 5 ml of absolute ethanol were performed in the same way (including boiling) and one extraction with 5 ml of ether was carried out at room temperature.

The precipitate was dissolved in formic acid (98-100%) by dropwise addition from a micropipette and 0.2 ml of the solution plated on weighed aluminum planchettes and counted.

17. Acid-Labile Fraction. Experiments in which extraction of an uncharacterized fraction containing terpene alcohols was made, followed the procedure for the extraction of fatty acids to the point of the third petroleum ether extraction. The combined petroleum ether extract (volume 30 ml) was vigorously extracted with 20 ml of normal sodium hydroxide three times, followed by three washes with distilled water of the same volume. The petroleum ether extract was dried with 5 mg of sodium sulfate and transferred to an 80 ml centrifuge tube containing 2 ml of toluene. The petroleum ether was removed under nitrogen at room temperature and to the residual 2 ml of toluene, 8 ml of scintillation fluid was added. The samples were counted in a liquid scintillation counter.

18. Recovery Experiments. Recovery experiments were performed by preparing the usual incubation medium including sodium acetate-1-C^{14}. To this medium was added a convenient amount of testosterone-4-C^{14} dissolved in 0.3 ml of propylene glycol and finally testis slices were added. The flask was immediately placed in a deep-freeze (-8°C) for the duration of the incubation. At the end of this time, the contents of the flask were allowed to thaw and were homogenized and extracted in the usual manner. The final chromatogram in ligroin-propylene-glycol was counted in the liquid scintillation counter together with aliquots of the original solution of testosterone-4-C^{14}.

19. Control Experiments. Control experiments included zero time controls in which the flasks were prepared in the usual way and kept

at -8°C for the duration of the incubation. In other control experiments a flask and its contents were heated at 46°C for 30 minutes, then incubated for two hours in the usual way. In all cases the control flasks were extracted in the same way as companion specimens.

20. Preincubation. In some experiments incubation was begun in the usual way and at certain time intervals was interrupted for the purpose of making some addition to or other change in the contents of the flask. The period prior to such interruption is referred to as the period of preincubation.

21. Protein Estimation. Estimation of tissue protein was performed by the Folin-Chiocalteau method (41). This method is a colorimetric procedure in which two colors are developed by the successive application of the biuret reaction and by the reduction of a phosphomolybdic-phosphotungstic reagent (Folin reagent) by the amino acids tyrosine and tryptophan. The assay was performed upon an aliquot of a solution of tissue in normal sodium hydroxide. The solution of tissue was prepared by dissolving the 0.1 ml sample removed after homogenizing the slices (see page 99) in 0.1 ml of normal sodium hydroxide, which was diluted to 1 ml by water after the tissue had dissolved.

To 0.1 ml of the dilute solution was added 1 ml of a reagent composed of a mixture of 0.5 per cent copper sulfate in 1 per cent sodium tartrate (1 ml) and 2 per cent aqueous sodium carbonate (49 ml). Ten minutes later, 0.1 ml of Folin reagent was added and after 30 minutes the solution was read against a reagent blank in a Coleman spectrophotometer at 750 mμ. A standard curve for this reaction was constructed from estimations performed on solutions of serum albumin.

22. Estimation of Cholesterol Fraction. Fractions C and D eluted from the aluminum oxide column described on page 100 were combined, dried under nitrogen and 5 mg of cholesterol added. The mixture was dissolved in 25 ml of a 1 per cent solution of digitonin in 90 per cent aqueous ethanol and warmed at 40°C until the solution was clear. This solution was left at 18°C overnight and then excess ether added. The resulting precipitate was washed three times with ether and dissolved in pyridine. The pyridine solution was plated and the planchette weighed and counted in a plate counter. The composition of the digitonin precipitable material was not studied in further detail.

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CHAPTER III

EXPERIMENTAL FINDINGS

Section 1

Stimulation *in vitro* of Slices of Rabbit Testis by Gonadotrophic Hormones.

Aims - 1. To demonstrate the influence of gonadotrophic hormones upon the rate of incorporation of sodium acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*.

2. To establish the identity of the radioactive testosterone resulting from 1.

3. To study the influence of substrate concentration and specific activity and of time, upon the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*.

1. The Influence of Gonadotrophic Hormones upon Slices of Rabbit Testis *in vitro*.

A. Chorionic Gonadotrophin. The effect of human chorionic gonadotrophin upon the incorporation of acetate-1-C^{14} into testosterone was tested by incubating slices of rabbit testis in the manner described on page 99. The contents of the incubation flasks were added in the following order:

Sodium Acetate	1.2×10^{-1} mMoles
Sodium Acetate-1-C^{14}	3.0×10^{-3} mMoles
HCG	5,000 I.U.
Krebs-Ringer bicarbonate buffer to	15 ml
Slices of Testis	1-3 Gm

The final specific activity of the sodium acetate was 0.05 mC per mMole. Incubation was for 6 hours at 37.5°C. Table XVI shows the results of such experiments.

Table XVI

The Influence of HCG upon the Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis in vitro

Experiment	Weight of Testis Gm/flask	HCG I.U./flask	Testosterone CPM
6	1.8	-	-
	1.6	-	-
	2.0	5,000	286
	1.2	5,000	190
9	1.2	-	-
	1.2	-	
	1.1	-	
	1.4	-	
	1.2	5,000	410
	1.0	5,000	
	1.1	5,000	
	1.7	5,000	

*Brackets indicate that specimens were pooled before measuring radioactivity.

Identification of Radioactive Material - The following criteria established the identity of the radioactive material isolated as testosterone: -

(i) The radioactive material behaved as testosterone on paper chromatography in ligroin-propylene glycol.

(ii) Acetylation followed by chromatography in hexano-benzene-formamide revealed that the acetylated radioactive compound behaved as testosterone acetate.

(iii) The acetate was recrystallized to constant specific activity following addition of 10 mg of crystalline testosterone acetate (Table XVII).

Table XVII

Recrystallization of Testosterone Acetate

Recrystallization	Solvent	DPM per mg
Original Mixture	Methanol	690
1st	Acetone	77
2nd	Ethanol	75
3rd	Acetone	70

B. Adenohypophysis. The anterior pituitary gland of an adult male dog was homogenized in Krebs-Ringer bicarbonate buffer and incubated with slices of rabbit testis as before; the results are shown in Table XVIII (Experiment 14).

Table XVIII

The Influence of Pituitary Homogenate on Slices of Rabbit Testis in vitro

Weight of Testis Gm/flask	Pituitary Homogenate	Testosterone CPM
2.4	-	-
2.8	+	600

Identification of Radioactive Material. Acetylation of the radioactive material followed by chromatography in hexane-benzene-formamide indicated that the acetate behaved as testosterone acetate in this system.

C. Follicle-Stimulating Hormone. The influence of FSH upon the incorporation of acetate-1-C^{14} into testosterone is shown in Table XIX.

Table XIX

The Influence of FSH on the Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis in vitro.

Experiment	Weight of Testis Gm/flask	FSH		Testosterone CPM
		Preparation	Weight mg/flask	
12	1.4	Steelman	-	-
	1.8		-	
	1.9		12.5	1,640
	1.6		12.5	
15	2.1	NIH (Vial)	-	-
	2.8		2.5	70
	3.1		5.0	142
	3.0		7.5	206
	2.9		10.0	394

26	2.0	NIH (Powder)	10	363
	2.0	(Vial)	10	352
	2.0	(Powder)	15	506
	2.0	(Vial)	15	484
	2.0	(Powder)	20	726
	2.0	(Vial)	20	748

* Brackets indicate that specimens were pooled before measuring radioactivity.

Identification of Radioactive Material. The following criteria extablished the identity of the radioactive material isolated, as testosterone.

(i) The radioactive material behaved as testosterone in the following systems of paper chromatography: -

(a) Ligroin - propylene glycol (moving 8 cm in 24 hours).

(b) Hexane - benzene - formamide (moving 11.5 cm in 2 hours).

(c) Bush A (moving 23 cm in 2½ hours).

(d) Cyclohexane - propylene glycol (moving 8.6 cm in 24 hours).

(ii) Acetylation followed by chromatography in hexane-benzene-formamide showed that the radioactive material behaved as testosterone acetate (moving 43 cm in 2 hours).

(iii) The acetate was recrystallized to constant specific activity (Table XX).

D. Interstitial Cell-Stimulating Hormone. The influence of ICSH (NIH-LH-S-1) upon the incorporation of acetate-1-C^{14} into testosterone is seen in Table XXI (Experiment 30).

Table XX

Recrystallization of Testosterone Acetate

Recrystallization	Solvent	DPM per mg
Original Mixture	Methanol	76
1st	Acetone	58
2nd	Ethanol	82
3rd	Methanol	81
4th	Ligroin	82

Table XXI

The Influence of ICSH and FSH upon the Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis *in vitro*.

FSH mg/flask	ICSH mg/flask	Weight of Testis per flask gm	Testosterone CPM	Cholesterol Fraction CPM*
-	-	1.0	242	1,676
0.1	-	1.0	4,620	1,806
0.5	-	1.0	2,160	2,274
1.0	-	1.0	3,180	2,425
5.0	-	1.0	4,000	2,517
-	0.5	1.0	14,080	2,486
0.5	0.5	1.0	6,460	2,290

* The "cholesterol fraction" consisted of the combined fractions C and D from the aluminum oxide column described on page 100. Between 68 and 80 per cent of the radioactivity of this fraction was precipitated by digitonin (page 111).

The identity of the radioactive material measured as testosterone in the case of ICSH stimulation was established as follows:

(i) The radioactive material behaved as testosterone in the following systems of chromatography.

(a) Ligroin-propylene glycol (moving 7.4 cm in 24 hours)

(b) Hexane-benzene-formamide (moving 11 cm in 2 hours).

(ii) Acetylation followed by chromatography in hexane-benzene-formamide revealed that the acetylated radioactive material behaved as testosterone acetate (moving 44 cm in 2½ hours in this system).

(iii) Oxidation with chromic acid followed by chromatography in hexane-benzene-formamide revealed that the oxidized radioactive material behaved as androstenedione (moving 14 cm in 2¼ hours in this system).

(iv) The original material, the acetate and the product of oxidation (androstenedione) were separately recrystallized to constant specific activity after addition of crystalline unlabeled carrier in each case (Table XXII).

(v) Material behaving as testosterone in ligroin-propylene glycol was eluted and eluates from six flasks were combined, dried under nitrogen and studied by gas chromatography. The combined specimen was shown to contain 0.06 µg of material which behaved as testosterone, showing a retention time of 15 minutes. In addition, a second compound was seen, showing a retention time of 11 minutes. The second compound has not been identified.

2. The system was studied with a view to finding the most suitable conditions of incubation in order to use the results as the basis for an assay system, measuring the rate of incorporation

Table XXII

Testosterone			Testosterone Acetate			Androstenedione		
Recrystal-lization	Solvent	DPM per mg	Recrystal-lization	Solvent	DPM per mg	Recrystal-lization	Solvent	DPM per mg
Original mixture	Methanol	233	Original mixture	Methanol	240	Original mixture	Methanol	180
1st	Ethanol	198	1st	Benzene	172	1st	Ethanol	184
2nd	Acetone	206	2nd	Ethanol	157	2nd	Methanol	164
3rd	Methanol	200	3rd	Ligroin	161	3rd	Acetone	184
4th	Benzene	206	4th	Methanol	164	4th	Methanol	181

of acetate-1-C^{14} into testosterone. The results of such experiments are shown in Table XXIII.

The following conclusions were drawn from these findings:-

1. More acetate was incorporated into testosterone in 3 hours than in 2 hours.

2. Experiment 28 suggests that a specific activity of approximately 7.3×10^{-2} µc per mMole of sodium acetate provided a response which could be conveniently measured as radioactive testosterone.

3. Experiment 29 suggests that 1.0×10^{-1} mMole of sodium acetate in 5 ml of medium almost saturates the system, since a three-fold increase in this concentration causes only a small increase in the response (a difference which is probably within the limits of experimental error).

It was apparent that slices of testis from different rabbits differed widely in their response to gonadotrophins and it proved difficult to mix slices from different animals to give a random mixture. It was, therefore, decided to reduce the volume of the medium to 3 ml and to conduct subsequent experiments in such a way that each experiment would be based upon the comparison of the activity of slices from one rabbit only. The system was accordingly modified as follows:

Weight of testis	0.5 gm
Final volume of medium	3.0 ml
Weight of acetate	5×10^{-2} mMole (8.5×10^{-2} µc/mMole)
Duration of incubation	2 hours

Since the identity of the radioactive material extracted following incubation was established as testosterone and since this material showed consistent behavior on paper chromatography in ligroin-propylene

Table XXIII

The Effect of Substrate Concentration, Specific Activity and Time upon the Rate of Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis.

Experiment	Wt of Testis* per flask gm	Volume of Medium ml	Duration of Incubation hours	Acetate mMoles per flask	Acetate µc per mMole	FSH mg/flask	Testosterone CPM
28	2.0	10.0	zero	3.3×10^{-2}	2.3×10^{-2}	12.5	-
	2.0	10.0	2	3.3×10^{-2}	2.3×10^{-2}	12.5	1,544
	2.0	10.0	2	3.3×10^{-2}	7.3×10^{-2}	12.5	2,627
	2.0	10.0	3	3.3×10^{-2}	2.3×10^{-2}	12.5	2,330
	2.0	10.0	3	3.3×10^{-2}	7.3×10^{-2}	12.5	5,390
29	1.0	5.0	2	3.3×10^{-2}	8.3×10^{-2}	5.0	850
	1.0	5.0	2	1.0×10^{-1}	8.3×10^{-2}	5.0	1,035
	1.0	5.0	2	3.0×10^{-1}	8.3×10^{-2}	5.0	1,220

* Slices from three rabbits were pooled and used in Experiment 28; slices from one rabbit were used in Experiment 29.

glycol, it was decided to measure the amount of radioactive testosterone formed in each experiment, by triangulation of the radioactive peak opposite the ultraviolet-absorbing carrier testosterone. A typical record of the radioactivity of such a chromatogram is shown in Figure 3.

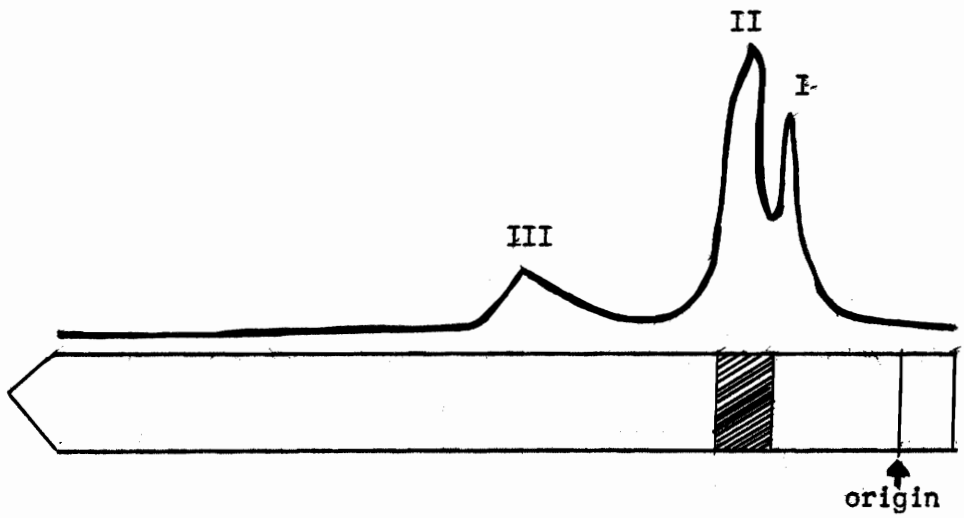


Figure 3. Tracing of the recorded radioactivity of a chromatogram developed in ligroin-propylene glycol from material extracted following incubation of slices of rabbit testis with sodium acetate-1-C^{14}. The shaded area represents the position occupied by carrier testosterone, as revealed by the Haines' scanner.

Peak I was studied by gas chromatography and shown to consist of at least 4 compounds, all of which behaved like steroids. One of these showed a retention time equal to that of pregnenolone, a second behaved like 17-α-hydroxypregnenolone and a third like 17-α-hydroxyprogesterone. The identification of these compounds was not studied in further detail.

Peak II was shown to consist of testosterone and an unidentified compound (page 122).

Peak III behaved like androstenedione on gas chromatography but has not been further identified.

Peak I could be further separated from testosterone by re-chromatography in ligroin-propylene glycol for 48 hours. Table XXIV shows the result of measurements of radioactivity by triangulation after the first (24 hours) chromatogram and after rechromatography (48 hours) in the same system.

Table XXIV

The Effect of Rechromatography on the Measurement of Radioactivity in Peak II by Triangulation.

Testosterone (CPM)	
First Chromatogram (24 hours)	Rechromatography (48 hours)
363	510
352	480
506	525
484	505
726	840
748	860

It was considered that the time required to effect this further separation of testosterone from peak I was not justified since the ratio of measured radioactivity between different specimens was not significantly altered. Rechromatography for shorter periods, extension of the original chromatogram to 48 hours, use of Bush A and

Zaffaroni systems after the first chromatogram, did not improve the separation of testosterone from peak I.

It is clear from the results of recrystallization that the radioactive testosterone isolated from the chromatograms was impure. The nature of the unidentified compound found on gas chromatography of material opposite peak II remains unknown, nor is it known whether this material is radioactive or not. Recent studies by Lipsky et al. showed that testosterone may decompose on gas chromatography(1), so that the substance mentioned may be a product of such decomposition.

Although it is realized that the testosterone isolated from such paper chromatograms may be impure, the total radioactivity measured by triangulation will be referred to as testosterone, for the sake of convenience and will be expressed as CPM per 100 mg (of testicular) protein.

3. In two experiments no incorporation of sodium mevalonate-2-C^{14} nor of cholesterol-4-C^{14} into testosterone by slices of rabbit testis was seen. It was not possible to demonstrate the incorporation of sodium butyrate-1-C^{14} into testosterone by slices of rabbit testis in concentrations of from 10^{-3} mMoles to 10^{-1} mMoles per flask. These substrates were tested both with and without ICSH.

Conclusions

These findings appear to justify the following conclusions:-

1. The hormones HCG, FSH and ICSH are capable of stimulating the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*. Homogenate of canine anterior pituitary gland is capable of causing the same response.

2. The identity of the testosterone so produced has been established.

3. Conditions of incubation suitable for measuring this response to gonadotrophic hormones have been established.

4. Slices of rabbit testis incubated in the absence of trophic hormones vary considerably in activity and frequently do not show detectable incorporation of acetate into testosterone.

5. The response of slices of rabbit testis to trophic hormones varies considerably from animal to animal.

6. Neither mevalonate, cholesterol nor butyrate could be shown to undergo incorporation into testosterone by slices of rabbit testis *in vitro*.

7. The radioactivity of an uncharacterized "cholesterol fraction" was not significantly altered by gonadotrophic hormones.

Reference

1. Lipsky, S. R. and Landowne, R. A. Effects of Varying the Chemical Composition of the Stationary Phase on the Separation of Certain C_{19}, C_{21}, and C_{27} Steroids by Gas Chromatography. Analytical Chemistry. 33:818,1961.

Section 2

The Response of Slices of Rabbit Testis to Trophic Hormones *in vitro*.

Aims - 1. To establish the lowest dose of FSH and ICSH to which slices of rabbit testis will respond *in vitro*.

2. To study possible synergism between FSH and ICSH in their action upon slices of rabbit testis *in vitro*.

3. To establish the specificity of the response of slices of rabbit testis to gonadotrophic hormones *in vitro*.

4. To make suitable control experiments.

5. To study the recovery of testosterone-4-C^{14} added to the incubation medium.

6. To study the influence of time upon the response of slices of rabbit testis to ICSH *in vitro*.

1. Experiments were designed to find the lowest dose of FSH and ICSH to which slices of rabbit testis would respond in the system described in Section 1. Table XXV shows the lowest dose of FSH to which slices of testis responded.

Table XXV

Response of Slices of Testis to FSH *in vitro*

Experiment	Lowest dose of FSH (µg) giving response	Testosterone CPM above control
32 *	5.0	660
34 *	1.0	341
38 *	10^{-3}	304
	10^{-3}	280
57	10.0	615

* Lower doses not tested.

Figure 4 shows the results of Experiments 35 and 36 in which the response of slices to low doses of ICSH was studied.

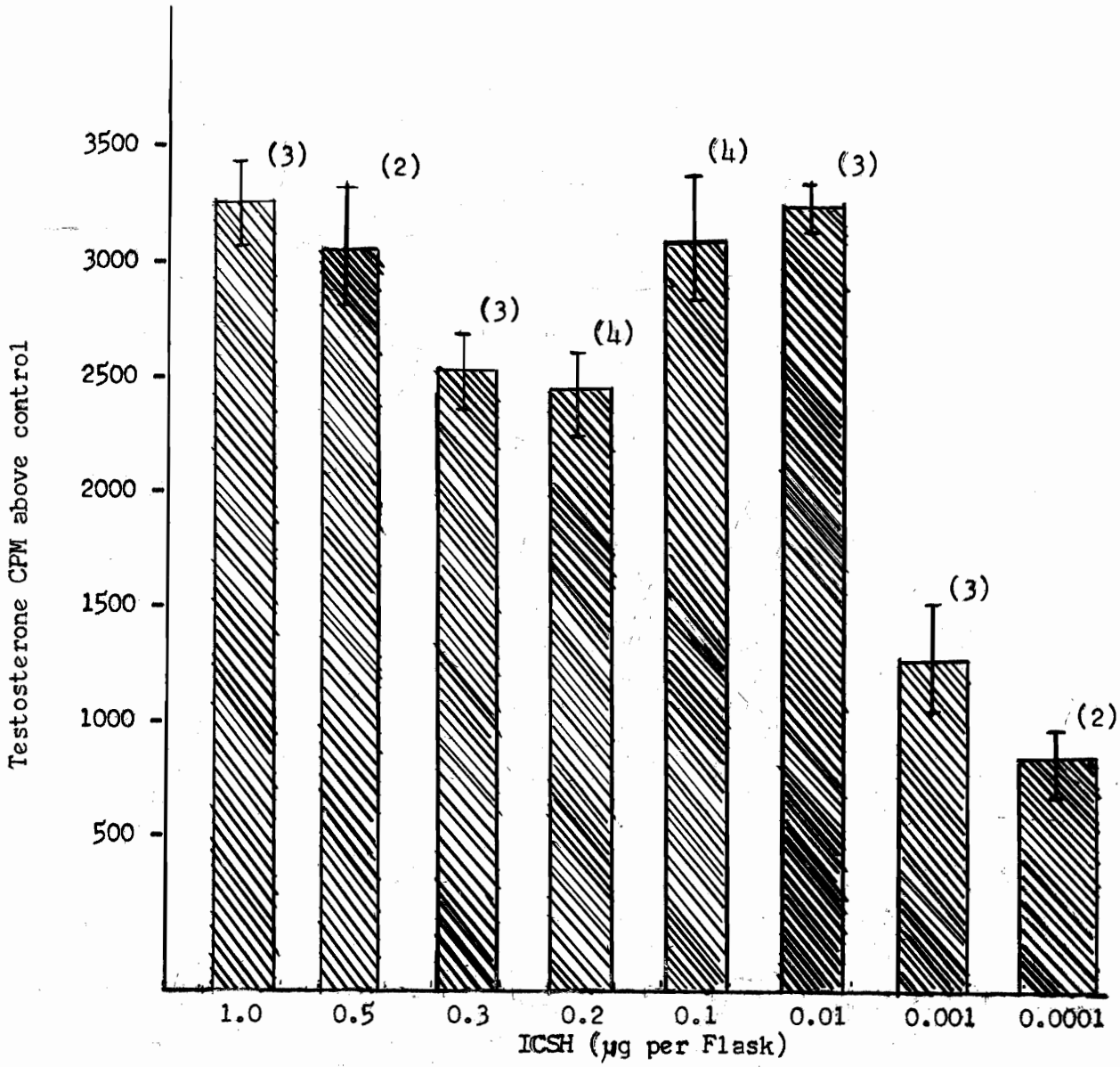


Figure 4. Response of slices of rabbit testis to ICSH. Numbers in parentheses indicate the number of observations. The mean is indicated by the bar and the range by I.

Table XXVI shows the lowest doses of ICSH which produced a response in slices of rabbit testis.

Table XXVI

Response of Slices of Testis to ICSH in vitro

Experiment	Lowest dose of ICSH (μg) giving response	Testosterone CPM above control
35 *	10^{-1}	2,884
36 *	10^{-4}	716
50 *	10^{-3}	238
55	10^{-3}	892
57	10^{-1}	1,063
76 *	10^{-3}	420

* Lower doses not tested.

2. In experiments 34, 55 and 57, doses of ICSH between 10^{-7} and 33 μg were incubated in the presence of doses of FSH between 10^{-5} and 500 μg in order to study the possible occurrence of synergism between the two hormones. In no case was a response obtained which was greater than the sum of the responses to either hormone alone.

Table XXVII shows the result of preincubating slices with one gonadotrophic hormone followed by addition of the other.

Table XXVII

The Effect of Preincubation upon the Joint Actions of FSH and ICSH on Slices of Rabbit Testis in vitro.

Experiment	Preincubation for 1st hour		Incubation for 2nd hour		CPM
	Hormone	Dose μg/flask	Hormone added	μg	Testoster
59	-	-	-	-	-
	ICSH	10^{-2}	-	-	-
	ICSH	10^{-2}	-	-	-
	FSH	5	-	-	-
	FSH	5	-	-	-
	ICSH	10^{-2}	FSH	5	616
	ICSH	10^{-2}	FSH	5	418
	FSH	5	ICSH	10^{-2}	200
	FSH	5	ICSH	10^{-2}	341

3. The specificity of the response of slices of testis to gonadotrophic hormones was demonstrated by failure of other pituitary hormones and of plasma protein to cause stimulation of slices in the system described in Section 1. Table XXVIII shows the substances tested. Slices of testis from the same animals responded to 10^{-3}µg ICSH per flask.

Table XXVIII

Substances which Failed to Stimulate Slices of Rabbit Testis in vitro.

Substance	Dose Tested per flask
TSH	1.0µg
ACTH	5.0 units
ADH	2.4×10^{-3} units
Growth Hormone	1.0µg
MSH	1.0µg
Plasma Protein	1.0µg
Prolactin	10^{-1} - 10.0µg

4. In eight experiments, flasks containing slices of rabbit testis were prepared in the usual way and one flask was kept at -6°C for the duration of incubation. In two experiments, flasks containing slices of rabbit testis were prepared and the contents heated at 40°C for 30 minutes, then incubated for two hours in the usual way. In every case these control flasks were shown to contain

no detectable radioactive testosterone.

5. Recovery experiments. The mean recovery of added testosterone-4-C^{14} in four experiments in which extraction included column chromatography, was 66 per cent. The mean recovery of added testosterone-4-C^{14} in five experiments without column chromatography was 67 per cent. The range of recovery in these experiments was 56 to 71 per cent.

6. Table XXIX shows the effect of time upon the response to ICSH of rabbit testis from one rabbit (Experiment 83).

Table XXIX

ICSH µg per flask	Time of incubation minutes	CPM Testosterone per 100 mg protein
10	30	-
10	60	-
10	90	2,993
10	120	3,512

Conclusions

These findings appear to justify the following conclusions:

1. The lowest doses of FSH and ICSH to which slices have been shown to respond were 10^{-3} µg and 10^{-4} µg respectively. The threshold dose varied considerably from animal to animal.

2. It was not possible to demonstrate synergism between FSH and ICSH when these hormones were added simultaneously but pre-incubation with either hormone, followed by addition of the other, gave a response with doses which were without effect by themselves.

3. The specificity of the response of slices of rabbit testis to gonadotrophic hormones was indicated by failure of slices to respond to other pituitary hormones and to plasma protein, in doses comparable with those which proved effective in the case of gonadotrophic hormones.

4. Control experiments performed at low and high temperatures suggest that enzymatic activity may be involved in the incorporation of acetate-1-C^{14} to testosterone by slices of rabbit testis <u>in vitro</u>.

5. Experiments with added testosterone-4-C^{14} indicate that the present method is capable of recovering approximately 66 per cent of the testosterone present, with or without column chromatography.

6. The occurrence of measurable stimulation of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis requires at least one hour of incubation in the present system.

In view of the fifth conclusion it was decided to omit column chromatography in subsequent experiments.

Section 3

The Influence of Hormones Administered *in vivo* upon the Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis *in vitro*.

Aim: To study the effect of pretreating rabbits *in vivo*, with HCG, FSH and testosterone upon the response of slices of rabbit testis to gonadotrophic hormones *in vitro*.

1. Rabbits were pretreated with HCG subcutaneously on alternate days, being given three injections, the last of which was administered two days before the experiment. The testes were removed and incubated in the usual way (page 99), without addition of gonadotrophic hormones. The dose of HCG used was 500 I.U. for each of the three injections. Table XXX shows the results of such experiments.

Table XXX

The Influence of HCG *in vivo* upon the Behavior of Slices *in vitro*

CPM Testosterone per 100 mg Protein					
Rabbits Pretreated with HCG			Untreated Rabbits		
No. of Rabbits	No. of Observations	CPM Testosterone (range)	No. of Rabbits	No. of Observations	CPM Testosterone (range)
11	15	1,397-5,612	22	30	0-836

2. Figure 5 shows the response of slices to a single intravenous injection of HCG, one testis having been removed before injection.

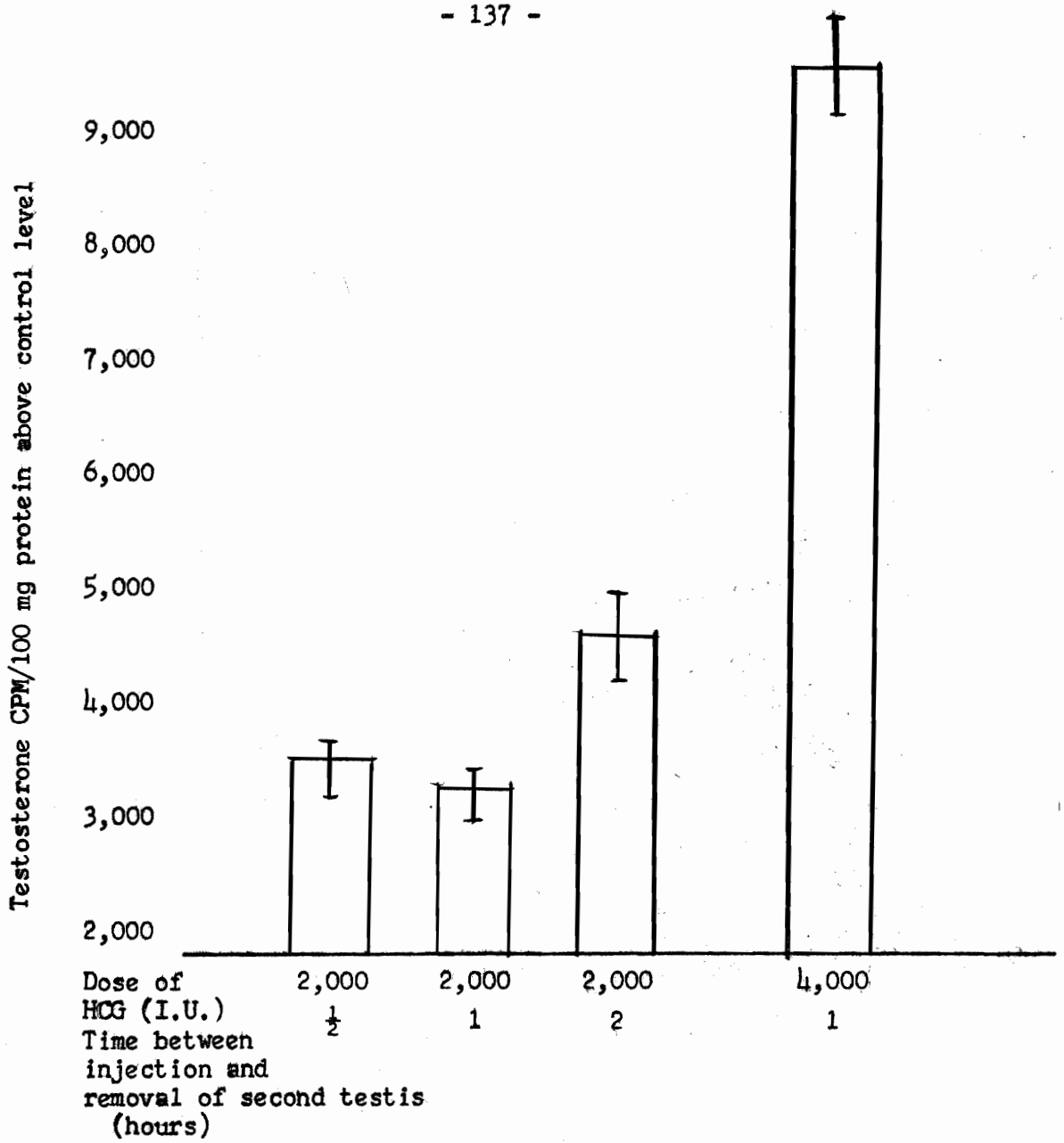


Figure 5. The increase in incorporation of acetate-1-C^{14} into testosterone in CPM per 100 mg protein above the pretreatment level, following a single intravenous injection of HCG. In each case the bar represents the mean of three readings, slices from one rabbit being used for each set of readings. The range is indicated by I .

3. Rabbits were pretreated with HCG subcutaneously on alternate days, being given three injections, the last injection two days before the experiment. The effect of FSH added *in vitro* to slices from animals pretreated in this manner, is seen in Table XXXI.

Table XXXI

The Effect of FSH *in vitro* on Slices of Rabbit Testis from Animals Pretreated with HCG *in vivo*.

Experiment	Pretreatment HCG I.U.	FSH µg/flask	Testosterone Slices without FSH expressed as 100
74	500x2, 1000x1	-	100
		10	36
75	500x2, 1000x1	-	100
		10^{-3}	91
		10^{-1}	69
		1.0	29
84	500x2, 1000x1	-	100
		10^{-2}	104
		10^{-1}	96
		1.0	32
		*10.0	30
		*10.0	33

* Puromycin 100 µg/ml added to this flask.

4. The specificity of this response to FSH was indicated by the effect of growth hormone, plasma protein and cold testosterone, shown in Table XXXII (Experiment 75).

Table XXXII

Pretreatment HCG I.U.	FSH µg/flask	Miscellaneous µg/flask	Testosterone (control as 100)
500x2, 1000x1	-	-	100
	10^{-3}	-	88
	10^{-1}	-	69
	1.0	-	29
	-	Growth Hormone 1.0	105
	-	Plasma Protein 1.0	102
	-	Testosterone 2.0	105

5. Table XXXIII shows the results of experiments in which FSH was added at different time intervals during the period of incubation.

Table XXXIII

Experiment	Pretreatment HCG I.U.	FSH µg/flask	FSH Time of addition after onset of incubation (minutes)	CPM Testosterone per 100 mg protein
75	500x3	-	-	6,317
		5.0	15	1,300
		5.0	30	1,422
		5.0	60	1,517
		5.0	90	1,623
84	500x2, 1000x1	-	-	1,984
		10.0	zero	901
		10.0	30	921
		10.0	60	884
		10.0	90	586

6. Tables XXXIV and XXXV show the effect of ICSH in vitro on slices of rabbit testis from animals pretreated with HCG.

Table XXXIV

Experiment	Pretreatment HCG I.U.	ICSH µg/flask	Testosterone (slices without ICSH expressed as 100)
63	500x3	-	100
		10^{-3}	86
		10^{-2}	200
		10^{-1}	210
73	500x3	-	100
		10^{-3}	150
		10^{-2}	146
		1.0	146

Table XXXV

Experiment	Pretreatment HCG I.U.	ICSH µg/flask	Testosterone slices CPM/100 mg protein
71	500x2,1000x1	-	1,328
		10^{-3}	1,320
		10^{-2}	1,296
		1.0	1,398
		10.0	1,247
75	500x2,1000x1	-	4,425
		10^{-3}	5,007
		10^{-1}	3,986
		10	4,392
	500x2,1000x1	-	9,147
		10^{-3}	7,856
		10^{-1}	8,319
		1.0	9,113

7. When pretreatment consisted of three injections each of 500 I.U. of HCG, doses of FSH (added *in vitro*), of 1 to 10 μg per flask, caused complete failure of detectable incorporation of acetate into testosterone.

8. Slices of testis from rabbits pretreated with FSH (5 mg x 3 subcutaneously, spaced as in the case of HCG) *in vivo*, failed to incorporate acetate-1-C^{14} into testosterone with or without addition *in vitro* of HCG in doses of 1 to 50 units or of ICSH in doses of 10^{-4} to 10 μg per flask.

9. Slices of rabbit testis from animals pretreated with testosterone (2.5 mg daily for 7 days) *in vivo*, failed to incorporate acetate-1-C^{14} into testosterone *in vitro* with or without ICSH in doses of 10^{-3} to 10 μg per flask.

Conclusions

These findings support the following conclusions:-

1. Pretreatment with HCG *in vivo*, stimulates the rate of incorporation of acetate-1-C^{14} into testosterone by slices of testis *in vitro*.

2. When pretreatment consists of three injections 500 I.U. of HCG each, slices are capable of showing further stimulation when ICSH is added *in vitro*, but when the pretreatment consists of two injections of 500 I.U. followed by one injection of 1000 I.U., no further response to ICSH *in vitro* is seen.

3. When pretreatment with HCG is followed by addition of FSH *in vitro*, decrease in the rate of incorporation of acetate-1-C^{14} is seen. This decrease is not significantly greater whether FSH is added at the beginning of incubation or as late as 30 minutes before it ends. The specificity of this response is shown by the fact that growth hormone, plasma protein, ICSH and testosterone are without such an effect. The inhibitory effect of FSH is not prevented by puromycin.

4. Pretreatment of rabbits with FSH renders slices of rabbit testis less responsive to ICSH *in vitro*.

5. Pretreatment of rabbits with testosterone renders slices of rabbit testis less sensitive to ICSH *in vitro*.

Section 4

Loss of Potency of ICSH Stored in Aqueous Solution.

Aims: 1. To compare the response of slices of rabbit testis to three different preparations of ICSH.

2. To study the deterioration of ICSH kept in aqueous solution.

1. Three preparations of sheep ICSH were available for testing (see page 96). The relative potencies of ICSH (Houston) and ICSH (NIH) had been compared with Armour standard LH #227-80, using the ventral prostate test in hypophysectomized rats and the Parlow ascorbic acid depletion tests (see page 96). In both tests ICSH (Houston) proved to be approximately twice as potent as ICSH (NIH). The three preparations were compared at two concentrations in the present system, as shown in Table XXXVI (Experiment 77). Slices of testis for this experiment were obtained from one rabbit.

Table XXXVI

Comparison of Three Preparations of ICSH in vitro

Preparation of ICSH	Dose µg/flask	CPM Testosterone per 100 mg Protein
Control	0	0
NIH	10^{-1}	5,765
Berkeley	10^{-1}	7,982
Houston	10^{-1}	6,102
NIH	10	12,022
Berkeley	10	11,084
Houston	10	9,862

2. The change in potency of solutions of ICSH (NIH) with time is shown in Table XXXVII (Experiments 58 and 80). Slices for each experiment were prepared from one rabbit.

Table XXXVII

Loss of Potency of ICSH in Aqueous Solution

Method of Storage (solutions contained 10 μg/ml in each case)	Time between preparation of solution and experiment (days)	Dose μg per flask	CPM Testosterone per 100 mg protein
Solution in water kept at 20°C	-	0	589
	-	10^{-1}	3,000
	3	10^{-1}	3,100
	8	10^{-1}	2,800
	10	10^{-1}	2,000
	16	10^{-1}	1,600
Frozen immediately after dissolving. Kept at -12°C; slowly thawed before use	-	0	-
	-	10^{x}	2,401
	25	10	-
	49	10	-

[x]Unfrozen fresh solution.

3. A whole anterior pituitary gland from an adult male dog which had been frozen for 6 months was thawed, homogenized and the homogenate divided equally between four flasks containing slices of rabbit testis, in the usual incubation medium. No incorporation of acetate-1-C^{14} into testosterone was found, although slices of testis from the same animal responded to ICSH (10^{-1} μg per flask).

Conclusions

These findings lead to the following conclusions:-

1. In the present system no significant difference in potency can be detected between ICSH (NIH), ICSH (Berkeley) and ICSH (Houston), in the two concentrations tested.

2. While solutions of ICSH (NIH) in water (1.0 µg/ml) at 20°C do not show gross deterioration within 3 days, loss of potency may appear after 8 days.

3. Frozen solutions of ICSH appear to lose potency after 25 days.

4. Prolonged freezing of anterior pituitary tissue appears to impair its gonadotrophic activity.

On the basis of these findings, it was considered that fresh solutions of ICSH should be used in subsequent experiments and unused solution discarded. It was also decided to use fresh pituitary tissue.

Section 5

The Influence of Hypothalamic Tissue upon the Release of Gonadotrophic Hormones from Pituitary Slices.

Aims: 1. To study the influence of homogenate of pituitary tissue upon the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis in vitro.

2. To study the rate of release of gonadotrophic hormones from slices of anterior pituitary gland with and without hypothalamic tissue in vitro.

3. To study the influence of vasopressin on the release of gonadotrophic hormones from slices of anterior pituitary gland in vitro.

1. Table XXXVIII shows the influence of pituitary homogenate upon the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis in vitro. Incubation was performed under standard conditions. The whole anterior pituitary gland (42 mg) of an adult male dog was homogenized and half of the resulting homogenate added to each of two flasks containing slices of rabbit testis, in the usual medium. Slices from one rabbit were used in this experiment (Experiment 44).

Table XXXVIII

Pituitary tissue mg per flask	ICSH µg per flask	CPM Testosterone
-	control	-
21	-	3,340
21	-	3,520
-	5×10^{-4}	832

2. Table XXXIX shows the results of experiments in which slices of anterior pituitary from 4 dogs were mixed and incubated with and without hypothalamic slices from one of these animals. Incubation lasted two hours; at the end of this time the pituitary slices were homogenized in Krebs-Ringer buffer (0.5 ml) and added to slices of rabbit testis in the usual medium. The medium in which the pituitary and hypothalamic slices had been incubated, was transferred to a flask containing slices of rabbit testis in the usual medium. In each case a second incubation of two hours duration was performed and the rate of incorporation of acetate-1-C^{14} into testosterone was measured.

Table XXXIX

Pituitary slices mg	Hypothalamic slices Site	mg	Ratio: Medium* / Pituitary
30	-		1.0
30	-		0.9
30	-		1.2
30	-		1.4
30	-		1.3
30	medium eminence	30	2.3
30	supraoptic nucleus	5	4.0
30	ventral median nucleus	30	3.5

* The ratio is calculated on the basis of the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis in the presence of the medium from the first incubation, over that found in the presence of the pituitary slices homogenized at the end of the first incubation.

3. Table XL shows the effect of vasopressin upon the distribution of gonadotrophic activity between pituitary slices and the surrounding medium. Incubation of pituitary tissue with and without vasopressin (0.1 units per flask) was conducted for 2 hours and the gonadotrophic activity in slices and medium compared by means of a second incubation with slices of rabbit testis and acetate-1-C^{14} as in 2 above.

Table XL

Pituitary slices mg/flask	Vasopressin units/flask	Ratio: $\frac{\text{Medium}}{\text{Pituitary}}$ *
20	-	0.35
23	-	0.4
20	0.1	0.38
23	0.1	0.05

* Calculated as in Table XXXIX.

Conclusions

These findings suggest that:-

1. Homogenate of anterior pituitary tissue is capable of increasing the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*.

2. Two hours of incubation of slices of anterior pituitary gland is accompanied by the release of gonadotrophic hormone from the pituitary slices into the surrounding medium.

3. Slices of hypothalamic tissue cause a significant increase in the release of gonadotrophin from slices of anterior pituitary tissue *in vitro*.

4. Vasopressin in the dose 0.1 units was without detectable effect upon the release of gonadotrophin from slices of anterior pituitary tissue *in vitro*.

Section 6

The Synthesis of Fatty Acids by Slices of Rabbit Testis.

Aims: 1. To study the fatty acid content of testicular and adrenal tissues.

2. To study the influence of ICSH upon the rate of incorporation of sodium acetate-1-C^{14} into fatty acids by slices of rabbit testis.

1. Slices of rabbit testis were incubated in the system already described with sodium acetate-1-C^{14} as substrate, both with and without ICSH (10 µg per flask). Slices of rabbit adrenal were preincubated with glucose 3 mg in 3 ml of Krebs-Ringer bicarbonate buffer, for one hour and then transferred to fresh buffer containing the same amount of C^{14}-labeled acetate as the flasks used for testicular slices. The incubation lasted two hours and was performed with and without ACTH (5 units per flask).

Following incubation the slices of testis and adrenal were homogenized and extracted with ether. The water phase was then extracted for fatty acids (page 106). The ether extracts were studied for steroid content by paper chromatography in ligroin-propylene glycol in the case of the testis and by the method of Peterson for the estimation of corticosterone (1), in the case of the adrenal. The fatty acid extracts were studied by gas chromatography on an ethylene-glycol-adipate column. The following fatty acids were isolated in the proportions shown:

Testis		Adrenal	
Fatty Acid	Ratio (oleate as 1)	Fatty Acid	Ratio (oleate as 1)
Palmitate	2.9	Palmitate	3.0
Palmitoleate	0.3	-	-
Stearate	0.9	Stearate	2.2
Oleate	1.0	Oleate	1.0

No significant difference was found between stimulated and unstimulated testis or adrenal, either in the amount or the proportion of the fatty acids isolated. The testicular slices showed increased incorporation of acetate-1-C^{14} into testosterone in the presence of ICSH and the adrenal slices showed greater incorporation of acetate-1-C^{14} into corticosterone in flasks containing ACTH.

It was not possible for measurements of the specific activities of these fatty acids to be made.

2. Slices of rabbit testis were incubated in the usual medium with and without ICSH and fatty acids extracted from the water phase after ether extraction. Figure 6 shows the result of one such experiment.

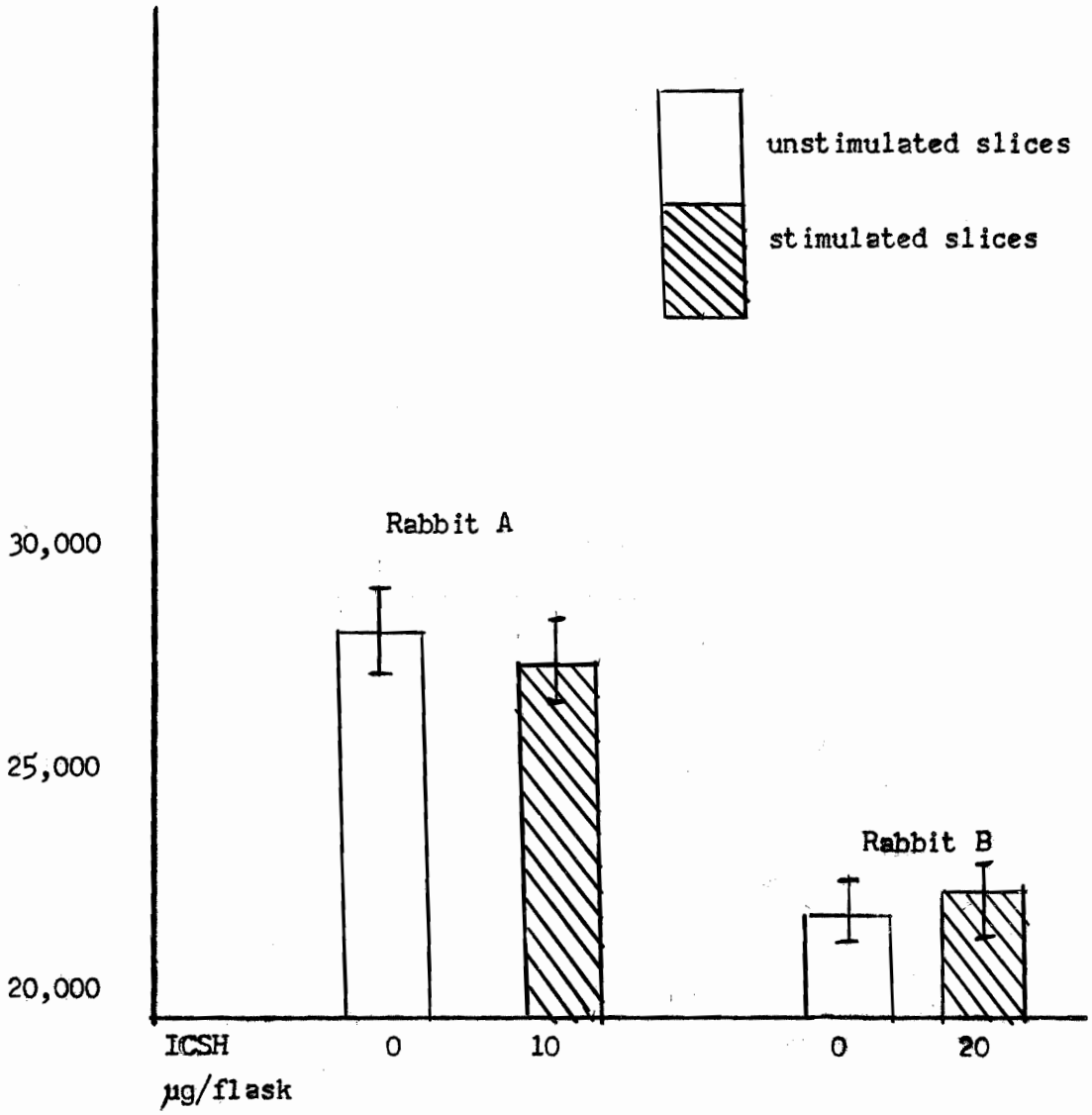


Figure 6. The incorporation of acetate-1-C^{14} into fatty acids by stimulated and unstimulated slices of rabbit testis. Stimulated slices showed an increase in the output of testosterone in both rabbits. Each bar represents the mean of three observations in each case and the rangeis indicated by I.

Conclusions

1. Rabbit testicular tissue contains palmitic, palmitoleic, stearic and oleic acids. Rabbit adrenal tissue contains palmitic, stearic and oleic acids.

2. The rate of incorporation of sodium acetate-1-C^{14} into an uncharacterized fatty acid fraction is not appreciably altered by concentrations of ICSH which stimulate the output of testosterone by slices of rabbit testis.

Reference

1. Peterson, R. E. Plasma Corticosterone and Hydrocorticosterone Levels in Man. J. Clin. Endocrinology. 17:1150,1957.

Section 7

The Influence of Pyridine Nucleotides, Cyclic AMP, Freezing, Glucose and Preincubation on the Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Rabbit Testis.

Aims: 1. To study the possible influence of pyridine nucleotides on the activity of slices of rabbit testis in vitro.

2. To study the possible influence of cyclic AMP on the activity of slices of rabbit testis in vitro.

3. To study the possible influence of:

(a) freezing,

(b) glucose and

(c) preincubation on the activity of slices of rabbit testis in vitro.

1. Addition of TPN in concentrations between 10^{-3}M and 10^{-2}M with and without a reducing system (glucose-6-PO_4 3 x 10^{-3}M and glucose-6-PO_4 dehydrogenase 50 µg) was without demonstrable effect upon slices of rabbit testis; slices from the same animals responded to ICSH (10 µg per flask).

Addition of DPN and DPNH in concentrations of $2.5x10^{-3}$M and $5x10^{-3}$M in each case, was without demonstrable effect upon the incorporation of acetate into testosterone by slices of rabbit testis; control slices responded to ICSH (10 µg per flask). Similarly, nicotinamide 0.2M and freezing of slices for 10 minutes followed by addition of 2.5×10^{-3}M of TPN with the reducing system described above and incubation in the usual way, were both without demonstrable effect on slices of testis.

2. The results of experiments in which cyclic AMP was added to the medium containing slices of rabbit testis, are shown in Table XLI (Experiments 46 and 58).

Table XLI

The Effect pf Cyclic AMP on Slices of Testis in vitro

LH (µg/flask)	Cyclic AMP	CPM Testosterone
-	-	-
10^{-3}	-	400
-	10^{-3}M	-
-	M	-
-	-	-
10^{-1}	-	1,036
-	10^{-3}M	-
-	$5x10^{-3}$M	-
-	10^{-2}M	-

3. The influence of freezing, of addition of glucose to the medium and of preincubation, on the activity of slices of rabbit testis in vitro, are shown in Table XLII.

Table XLII

The Effects of Freezing, Glucose and Preincubation on the Activity of Slices of Rabbit Testis in vitro.

Experiment	Conditions	Addition	Testosterone CPM
66	Standard +	-	-
	Standard	ICSH 10^{-3} µg	858
	Slices and medium frozen for 10 minutes, thawed, followed by addition of - - - - - - - - - - - -	ICSH 10^{-3} µg	851
79	Standard	-	-
	Standard	ICSH 10 µg	660
	Slices and medium frozen for 10 minutes, thawed and incubated under standard conditions	-	-
	Slices and medium frozen for 10 minutes, thawed, followed by addition of - - - - - - - - - - - -	TPN 2.5×10^{-3}M + reducing system	-
76	Standard	-	-
	Standard	ICSH 1 µg	2,231
	Glucose (3 mg) added to medium	ICSH 1 µg	1,924
	One hour preincubation with substrate, then addition of - - - - -	ICSH 1 µg	2,220
	One hour preincubation with buffer only followed by addition of fresh buffer and - - - - - - - -	ICSH 1 µg	896
	One hour preincubation with substrate, followed by addition of fresh buffer and - - - - - - - - -	ICSH 1 µg	2,157
62	Standard	-	-
	Standard	ICSH 1 µg	200
	One hour preincubation with substrate, buffer and ICSH 1 µg followed by addition of - - - - - -	TPN 1.0×10^{-3}M + reducing system	-
	The same preincubation	TPN 2.5×10^{-3}M + reducing system	-
	The same preincubation	TPN 5.0×10^{-3}M + reducing system	-
	One hour preincubation with TPN (2.5×10^{-3}M) and reducing system, followed by addition of - - - - - -	ICSH 1 µg	-

+Standard conditions are those described on page 99. In each case, preincubation or freezing and thawing was followed by two hours incubation under standard conditions.

Conclusions

These findings lead to the following conclusions:

1. In the concentrations studied, pyridine nucleotides (oxidized or reduced) have failed to stimulate theincorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*. Previous freezing of slices did not cause a response to the addition of reduced TPN.

2. Cyclic AMP in concentrations of 10^{-3}M to molar, did not stimulate the incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*.

3. Brief freezing of slices of rabbit testis did not stimulate the incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*, nor did such treatment alter the response to 10^{-3} µg ICSH.

4. Glucose in the concentration of 1 mg per ml did not alter the response of slices of rabbit testis to 1.0 µg ICSH.

5. Preincubation with acetate-1-C^{14} did not affect the response of slices of rabbit testis *in vitro* to ICSH (1 µg per flask), whether the subsequent incubation was performed in the same substrate and buffer or whether the slices were transferred to fresh medium. Preincubation in buffer alone followed by addition of substrate and ICSH, appeared to cause a diminished response to ICSH.

Section 8

The Activity of a Subcellular Preparation of Testicular Tissue.

Aim: To study the activity of a homogenate of rabbit testis with respect to:

1. Conversion of acetate, mevalonate and cholesterol to testosterone.

2. Possible response to gonadotrophic hormones.

3. Possible influence of ICSH on the conversion of pregnenolone-7-H^3 to testosterone.

4. Possible influence of FSH on the conversion of cholesterol-4-C^{14} to testosterone in animals pretreated with HCG in vivo.

1. Attempts to demonstrate the conversion of sodium acetate-1-C^{14} to testosterone by homogenate of rabbit testis failed; the following conditions were tested:

(a) Homogenate of testes from untreated animals.

(b) Addition of ICSH in doses of 10^{-4} to 5×10^{-1} µg per flask and FSH in doses of 5 mg per flask.

(c) Homogenate of testes from rabbits pretreated with HCG (three subcutaneous injections of 500 I.U. each according to the scheme described on page 86).

(d) Slices of testis from untreated animals were preincubated in buffer with ICSH (5×10^{-1} µg per flask) for one hour. The slices were then homogenized and added to acetate-1-C^{14} in fresh buffer.

(e) Homogenate of testes from rabbits which had been given a single intravenous injection of HCG (3,000 I.U.), one hour before removal of the testes.

(f) Whole homogenate of testes from untreated rabbits.

In these experiments the medium and method of incubation were as described on page 89. Homogenates were centrifuged as described on page 87 , except in cases where whole homogenate was used. Flasks were prepared by adding the following substances in the concentrations shown:

ATP	1×10^{-3}M
DPN	1.5×10^{-3}M
TPN	1.5×10^{-3}M
Glucose-6-PO_4	3×10^{-3}M
Glucose-6-PO_4 dehydrogenase	50 µg
Homogenate	0.5 ml
Phosphate buffer to a final volume of 3 ml	

2. In the experiments described in 1, the total radioactivity found in the crude "cholesterol" fraction (page 111), was less than 92 CPM above background in every case.

3. Attempts to demonstrate the conversion of mevalonate-2-C^{14} and butyrate-1-C^{14} to testosterone under the conditions enumerated in 1, were uniformly without success. Addition of extra TPN to a concentration of 1.5×10^{-2}M per flask with either acetate-1-C^{14}, mevalonate-2-C^{14} or butyrate-1-C^{14} as substrate failed to produce demonstrable incorporation of C^{14} into testosterone.

4. Figure 7 shows the effect of a single intravenous injection of HCG (2,000 I.U.) upon the rate of incorporation of cholesterol-4-C^{14} into testosterone by homogenate of rabbit testis. The left testis was removed before injection and the right testis was removed one hour after injection.

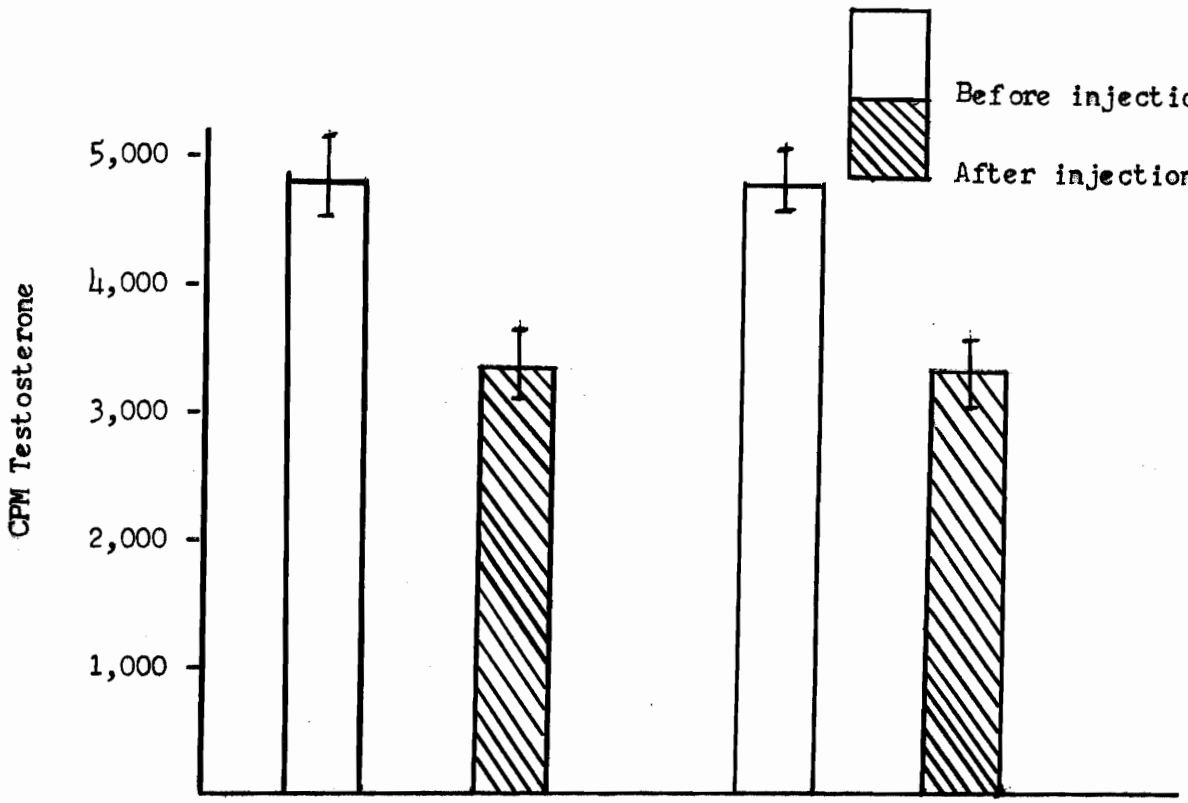


Figure 7. The influence of a single intravenous injection of HCG (2,000 I.U.) in vivo, on the conversion of cholesterol-4-C^{14} to testosterone by homogenate of rabbit testis. Each bar represents the mean of duplicate flasks, the range being indicated by I.

5. Homogenate of rabbit testis from which cell debris was removed by centrifugation at 650 x g for 15 minutes, proved capable of converting cholesterol-4-C^{14} to testosterone as shown in Table XLIII.

Table XLIII

The Conversion of Cholesterol-4-C^{14} to Testosterone by Homogenate of Rabbit Testis.

Experiment	Cholesterol Weight (µg/flask)	Cholesterol Specific Activity µc/mg	ICSH µg/flask	FSH µg/flask	Testosterone CPM
31	3	29	-	-	370
			5×10^{-2}	-	337
43	16	50	-	-	686
			5×10^{-2}	-	647
54	16	50	-	-	1,340
			10^{-1}	-	1,074
56	16	50	-	-	537
			1.0	-	276
66	280	25	-	-	1,403
			-	-	1,465
			10^{-3}	-	1,267
			-	1.0	1,379
79	60	25	-	-	2,970
			1.0	-	2,587
			10.0	-	3,194
			20.0	-	3,155
			+ -	-	3,108
			* -	-	3,164

+ Versene and cysteine were omitted from the buffer in which the homogenate was prepared.

* Nicotinamide was omitted from the medium in which the homogenate was prepared.

6. Table XLIV shows the effect of FSH in vitro upon the conversion of cholesterol-4-C^{14} to testosterone by homogenate of testes from rabbits treated with HCG (two injections of 500 I.U. each and one of 1,000 I.U. spaced as described on page 86). Experiment 82.

Table XLIV

Cholesterol-4-C^{14}		FSH	Testosterone
Weight (µg/flask)	specific activity (µc/µg)	(µg/flask)	CPM
60	50	-	2,218
		-	1,855
		10^{-1}	1,848
		10.0	2,178

7. Table XLV shows the effect of adding TPN (with a reducing system) and cyclic AMP to a homogenate of rabbit testis, upon its capacity to convert cholesterol-4-C^{14} to testosterone.

Table XLV

Experiment	Cholesterol		TPN*	Cyclic AMP	Testosterone
	Weight (µg/flask)	Specific activity (µc/µg)			CPM
79	60	25	-	-	825
			-	-	762
			2.5×10^{-3}M	-	2,970
			2.5×10^{-3}M	-	3,194
100	60	25	-	-	975
			-	-	867
			1.0×10^{-3}M	-	3,208
			2.5×10^{-3}M	-	3,478
			2.5×10^{-3}M	-	3,445
			5.0×10^{-3}M	-	2,132
46	16	50	1.5×10^{-3}M	-	524
			1.5×10^{-3}M	1.0×10^{-3}M	427
			1.5×10^{-3}M	5.0×10^{-3}M	468

* All flasks contained glucose-6-phosphate 3×10^{-3}M and glucose-6-phosphate dehydrogenase 50 µg.

8. In experiment 79 it was further shown that ICSH in doses of 1.0 and 10 μg per flask, did not increase the rate of conversion of cholesterol-4-C^{14} to testosterone.

9. The identity of the radioactive material isolated in these experiments was established as follows:

(a) The radioactive compound and the carrier testosterone moved the same distance in the paper chromatographic systems ligroin-propylene glycol and hexane-benzene -formamide.

(b) Acetylation followed by paper chromatography in hexane-benzene-formamide, revealed that the acetylated radioactive compound behaved in the same way as testosterone acetate.

(c) Oxidation by chromic acid, followed by chromatography in hexane-benzene-formamide, showed that the oxidized radioactive compound behaved in the same way as androstenedione.

(d) Table XLVI shows the result of recrystallization of testosterone, testosterone acetate and androstenedione eluted from the chromatograms described in (a), (b) and (c) above.

10. The conversion of Δ^5-pregnenolone-7-H^3 to testosterone by homogenate of testis with and without ICSH is shown in Table XLVII.

The identity of the tritiated compound as testosterone was indicated by:

(a) the fact that it behaved in the same manner as carrier testosterone on paper chromatography in the system ligroin-propylene glycol and

(b) that the acetate behaved as testosterone acetate in hexane-benzene-formamide.

Table XLVI

Recrystallization of Testosterone, Testosterone Acetate and Androstenedione

Testosterone			Testosterone Acetate			Androstenedione		
Recrystallization	Solvent	DPM per mg	Recrystallization	Solvent	DPM per mg	Recrystallization	Solvent	DPM per mg
Original mixture	Methanol	130	Original mixture	Methanol	174	Original mixture	Methanol	160
1st	Ethanol	100	1st	Acetone	148	1st	Ethanol	112
2nd	Acetone	91	2nd	Ethanol	142	2nd	Acetone	116
3rd	Methanol	88	3rd	Acetone	146	3rd	Benzene	114
4th	Benzene	94	4th	Methanol	140	4th	Ethanol	109

Table XLVII

Conversion of Pregnenolone-7-H^3 to Testosterone by Homogenate of Testis

Experiment	Pregnenolone-7-H^3 Weight (µg/flask)	Specific Activity µc/mg	ICSH µg/flask	CPM Testosterone Acetate	CPM Testosterone
51	0.5	600	-	2,634	
	0.5	600	10^{-3}	3,211	
	2.5	600	-	7,924	
	2.5	600	10^{-3}	7,499	
54	0.5	600	-	2,049	
	0.5	600	10^{-1}	2,770	
	2.5	600	-	7,809	
	2.5	600	10^{-1}	6,125	
56	0.5	600	-	3,033	
	0.5	600	1.0	4,413	
	0.5	600	-	-	6,268
	0.5	600	1.0	-	8,389
	1.25	600	-	-	20,342
	1.25	600	1.0	-	18,347
	2.5	600	-	-	35,118
	2.5	600	1.0	-	32,540

In Experiments 51 and 54 acetylation was performed to assist in identifying the tritiated compound; the acetate was counted in the liquid scintillation counter after chromatography in hexane-benzene-formamide. In Experiment 56 duplicate specimens were counted as testosterone in the liquid scintillation counter, to see whether losses during acetylation had obscured a significant difference between stimulated and unstimulated homogenate.

Conclusions

These findings lead to the following conclusions:

1. Homogenate of rabbit testis is capable of converting cholesterol-4-C^{14} to testosterone but neither sodium acetate-1-C^{14} nor sodium mevalonate-2-C^{14} nor sodium butyrate-1-C^{14} was converted to testosterone.

2. Neither stimulation *in vivo* with HCG, nor addition of gonadotrophic hormones *in vitro*, nor preincubation of slices with ICSH, caused homogenate of rabbit testis to convert acetate-1-C^{14} or mevalonate-2-C^{14} to testosterone. Tenfold increase in the concentration of TPNH added to the homogenate did not cause incorporation of these substrates into testosterone.

3. Neither stimulation *in vivo* with HCG nor addition *in vitro* of gonadotrophic hormones to homogenate of rabbit testis caused stimulation of the rate of conversion of cholesterol-4-C^{14} to testosterone. Omission of versene and cysteine or of nicotininamide, did not affect the activity of a testicular homogenate.

4. FSH *in vitro* did not cause inhibition of the rate of conversion of cholesterol-4-C^{14} to testosterone by homogenate of rabbit testis from animals pretreated with HCG *in vivo*.

5. Cyclic AMP did not enhance the rate of conversion of cholesterol-4-C^{14} to testosterone by homogenate of rabbit testis.

6. Addition of TPN and a reducing system enhanced the rate of conversion of cholesterol-4-C^{14} to testosterone by homogenate of rabbit testis.

7. The identity of the testosterone synthesized in these experiments has been established by the chromatographic behavior and by the recrystal-

lization to constant specific activity of the original compound and of two derivatives.

8. Homogenate of rabbit testis converted Δ^{5} pregnenolone-7-H^{3} to testosterone but addition of ICSH <u>in vitro</u> did not stimulate the rate of this conversion.

Section 9

The Effect of Gonadotrophic Hormones on the Permeability of Testicular Cells.

Aim: To study the possible influence of ICSH and FSH upon the permeability of testicular cells in vitro.

1. Table XLVIII shows the results of experiments in which the usual incubation of slices of rabbit testis was performed in the presence of D-xylose-1-C^{14}, but without acetate-1-C^{14}. The results are expressed as counts per minute per 100 mg dry weight of tissue. The slices were washed to remove adherent D-xylose and the radioactivity extracted by boiling the slices was considered to reflect the amount of D-xylose-1-C^{14} which had entered the cells (page 107).

2. Table XLIX shows the result of a similar experiment using α-aminoisobutyric acid-1-C^{14} instead of D-xylose-1-C^{14}.

In these experiments the tissue remaining after extraction with boiling water was dried at 120°C and counted in the liquid scintillation counter. In each case the total radioactivity was less than 50 counts per minute above background, indicating that the boiling water extracted all but an insignificant amount of the radioactive material.

Table XLVIII

The Effect of Gonadotrophins on the Permeability of Testicular Cells to D-xylose-1-C^{14}.

Experiment	D-xylose-1-C^{14} µg/flask	D-xylose-1-C^{14} µc/ug	Duration of Incubation	FSH µg/flask	ICSH µg/flask	CPM/100 mg dry weight of tissue
78	0.5	$4.1x10^{-3}$	2 hours	-	-	287
	0.5	$4.1x10^{-3}$	2 hours	-	100	294
	5	$4.1x10^{-3}$	2 hours	-	-	963
	5	$4.1x10^{-3}$	2 hours	100	-	2,138
	50	$4.1x10^{-3}$	2 hours	-	-	8,992
	50	$4.1x10^{-3}$	2 hours	100	-	10,500
	50	$4.1x10^{-3}$	2 hours	-	25	5,335
	50	$4.1x10^{-3}$	2 hours	100	-	5,428
81	150	$4.1x10^{-3}$	zero time	-	-	4,815
	150	$4.1x10^{-3}$	zero time	25	-	4,985
	150	$4.1x10^{-3}$	10 minutes	-	-	8,940
	150	$4.1x10^{-3}$	10 minutes	25	-	10,410
	150	$4.1x10^{-3}$	2 hours	-	-	45,825
	150	$4.1x10^{-3}$	2 hours	25	-	50,640
	150	$4.1x10^{-3}$	2 hours	-	-	42,555
	150	$4.1x10^{-3}$	2 hours	-	10	59,355
	150	$4.1x10^{-3}$	2 hours	-	25	43,200
	150	$4.1x10^{-3}$	2 hours	ACTH	10 units	30,390

				FSH µg/flask	ICSH µg/flask	
Slices of rabbit kidney	150	$4.1x10^{-3}$	2 hours	-	-	30,560
	150	$4.1x10^{-3}$	2 hours	25	-	31,680
	150	$4.1x10^{-3}$	2 hours	-	25	28,725

Table XLIX

The Effect of Gonadotrophins on the Permeability of Testicular Cells to ∝-amino-isobutyric acid-1-C^{14}

Experiment	-aminoisobutyric acid-1-C^{14} µg/flask	µc/ug	Time of incubation	FSH µg/flask	ICSH µg/flask	CPM/100 mg dry weight of tissue
88	150	0.028	zero time	-	-	3,536
	150	0.028	zero time	-	25	3,520
	150	0.028	zero time	25	-	2,808
	150	0.028	5 minutes	-	-	7,096
	150	0.028	5 minutes	-	25	6,862
	150	0.028	5 minutes	25	-	5,700
	150	0.028	15 minutes	-	-	16,436
	150	0.028	15 minutes	-	25	19,932
	150	0.028	15 minutes	25	-	17,496
	150	0.028	45 minutes	-	-	26,208
	150	0.028	45 minutes	-	25	24,532
	150	0.028	45 minutes	25	-	23,808

Conclusion

These findings lead to the conclusion that under the conditions tested, ICSH and FSH have not been shown to exert a significant and reproducible effect upon the transport of D-xylose-1-C^{14} or α-amino-isobutyric acid-1-C^{14} into the cells of the testis.

Section 10

The Role of Protein Synthesis in the Response of Slices of Testis to ICSH.

Aims: 1. To study the influence of inhibitors of protein synthesis on the response of testicular slices to ICSH *in vitro*.

2. To study the influence of Puromycin, administered *in vivo*, upon the response of the testis to HCG *in vivo*.

3. To study the effect of HCG and ICSH *in vivo* upon the rate of incorporation of labeled amino acids into protein by slices of rabbit testis *in vitro*.

4. To study the effect of ICSH upon the rate of incorporation of labeled amino acids into protein by slices of rabbit testis *in vitro*.

1. Table L shows the results of experiments in which chloramphemicol and puromycin were added to slices of rabbit testis incubated with and without ICSH. (Experiments 47,58,80,82,95).

2. Table LI shows the result of an experiment in which puromycin and chloramphemicol were added to slices of rabbit testis from a rabbit pretreated with HCG *in vivo* (Experiment 82).

3. In Experiment 87 two rabbits were anesthetized and the left testis of each removed and slices were incubated with sodium acetate-1-C^{14} in the usual system. Rabbit A was given a single intravenous injection of HCG (1,000 I.U.), rabbit B was given puromycin (100 mg per kg body weight) intravenously, followed by HCG (1,000 I.U.). One hour later the right testis was removed from each rabbit and slices were incubated as before. The mean production of labeled testosterone

Table L

The Influence of Chloramphemicol and Puromycin upon the Response of Slices of Testis to ICSH.

ICSH µg/flask	Chloramphemicol µg/ml	Puromycin µg/ml	Percent Stimulation[x]
10^{-3}	-	-	100
10^{-3}	10	-	113
10^{-1}	-	-	100
10^{-1}	10	-	70
10^{-1}	100	-	0
10	-	-	100
10	10	-	114
10	100	-	0
10	-	-	100
10	-	10	32
10	-	100	0
10	-	1	130
10	-	5	124
50	-	-	100
50	-	50	0
50	-	100	0

[x] The increase in incorporation of acetate-1-C^{14} into testosterone above the control (unstimulated) level, caused by the addition of ICSH is expressed as 100.

Table LI

HCG Pretreatment I.U.	Puromycin µg/ml	Chloramphemicol µg/ml	Testosterone CPM/100mg Protein
500 x 2; 1000 x 1	-	-	5,612
	10	-	5,264
	100	-	5,500
	-	100	5,731

above the control level following injection, was for rabbit A 8,924 CPM per 100 mg protein and 3,541 for rabbit B.

4. Figure 8 shows the results of experiments in which the rate of incorporation of tryptophan-1-C^{14} into protein was measured before and after injection of HCG.

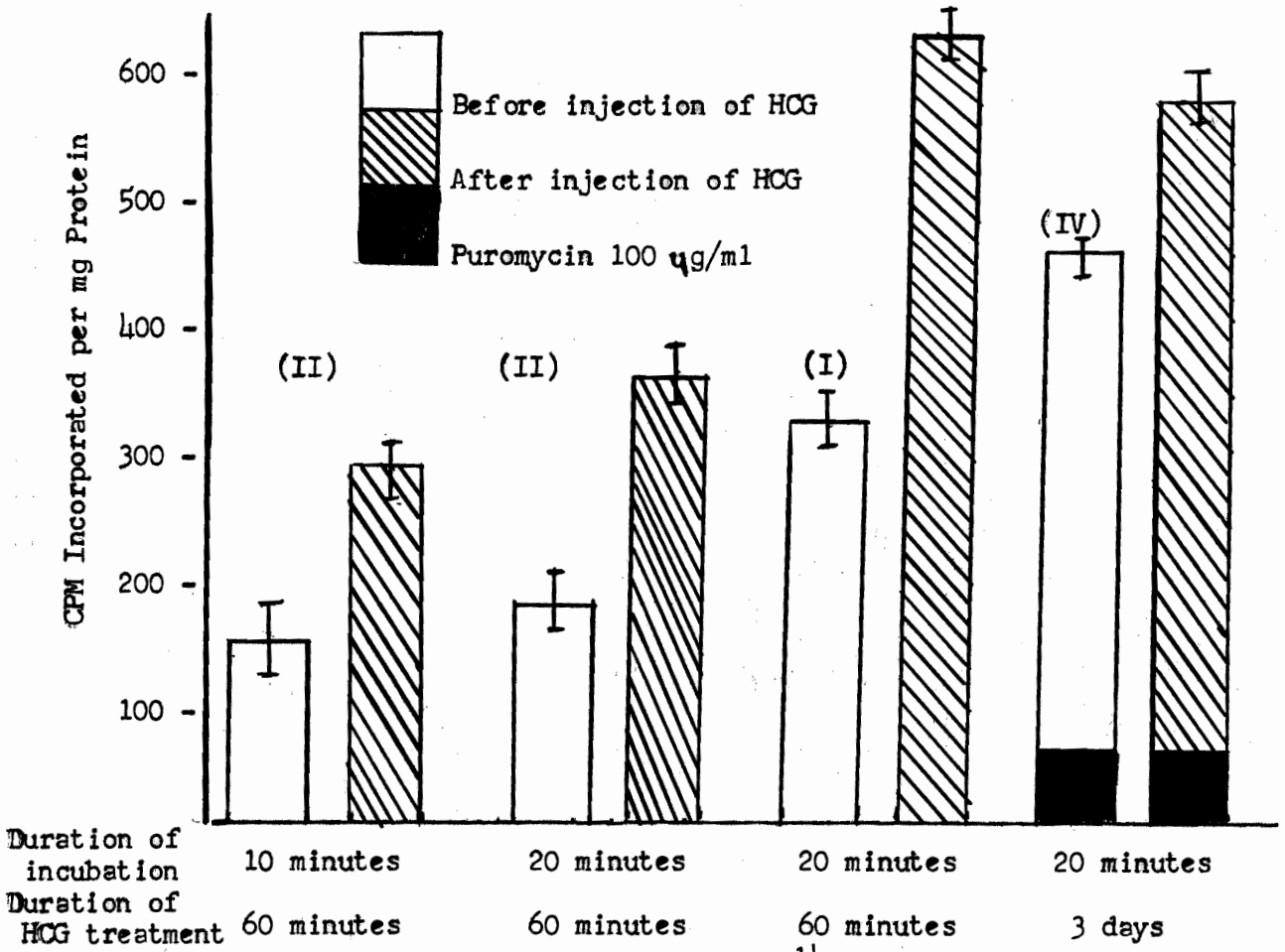


Figure 8. Incorporation of tryptophan-1'-C^{14} into protein before and after stimulation with HCG. Treatment in vivo for 60 minutes was achieved by a single intravenous injection of HCG (1,000 I.U.), immediately after removal of the control testis. Treatment for 3 days was achieved by three daily subcutaneous injections of HCG (1,000 I.U. each), the first being given immediately after removal of the control testis and the third being given 24 hours before removal of the second testis. Figures in brackets indicate the number of animals used. Each bar represents the mean of 4 flasks from each animal and the range is indicated by I. Tryptophan-1'-C^{14} 2.3×10^{-4} mMoles was added to each flask (specific activity 3.8mc per mMole). The word "protein" is used to describe the material precipitated by TCA under the conditions described on page 108.

5. Stimulation in vivo for 3 and 10 minute intervals by means of a single intravenous injection of HCG (1,000 I.U.) produced no significant change in the rate of incorporation of tryptophan into protein.

6. Figure 9 shows the influence of ICSH in vivo on the rate of incorporation of tryptophan-1'-C^{14} into protein by slices of rabbit testis in vitro.

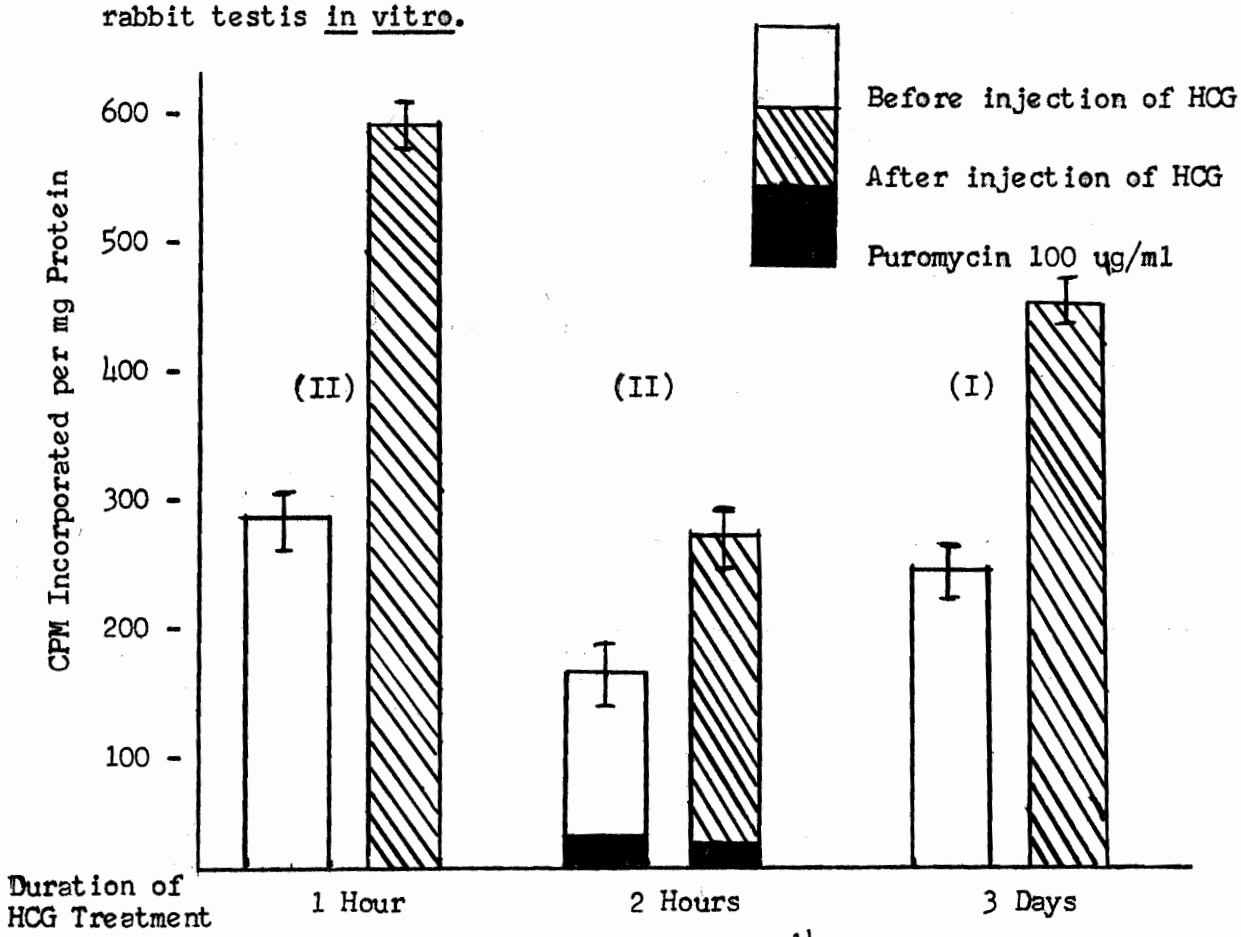


Figure 9. Incorporation of tryptophan-1'-C^{14} into protein before and after treatment with ICSH. Treatment in vivo for one and two hours was by means of a single intravenous injection; treatment for 3 days followed the method given in the caption to figure 8, except that the dose of ICSH was 1 mg for each injection. Experimental details and method of presenting data are exactly as for figure 8, except that in every case incubation was for 20 minutes.

7. In the experiment involving treatment with ICSH for one hour reported in figure 9, the rate of incorporation of tryptophan-1'-C^{14} into protein by slices of kidney was not significantly altered following in-

8. Figure 10 shows the results of experiments in which incorporation of valine-1-C^{14} into protein by slices of rabbit testis with and without ICSH in vitro was studied.

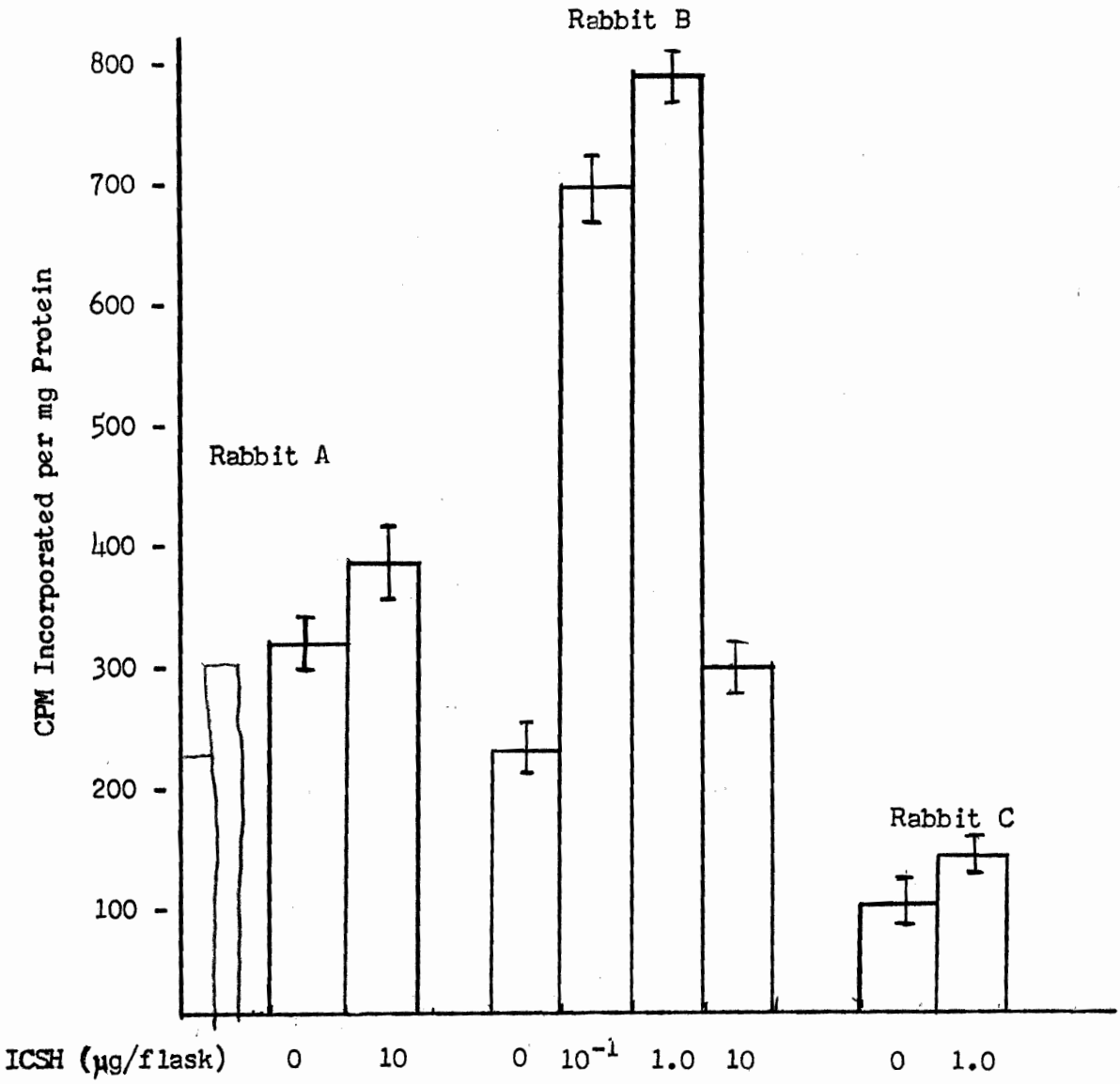


Figure 10. Incorporation of valine-1-C^{14} into protein by slices of rabbit testis in vitro, with and without ICSH. Incubation lasted 20 minutes and each flask contained valine-1-C^{14} 2×10^{-4} mMoles (specific activity 6 mc per mMole). Each bar represents the mean of duplicate flasks, the range is indicated by I.

9. Table LII shows the influence of ICSH upon the incorporation of tryptophan-1'-C^{14} into protein by slices of rabbit testis *in vitro*. Incubation was for 20 minutes and to each flask tryptophan-1'-C^{14} 2.3×10^{-4} mM (specific activity 3.8 mc per mMole) was added.

Table LII

The Effect of ICSH Added *in vitro* upon Incorporation of Tryptophan-1-C^{14} into Protein by Slices of Rabbit Testis.

ICSH µg/flask	Puromycin µg/ml	CPM/mg Protein
-	-	237
-	-	228
10	-	296
10	-	301
10	100	82

In this experiment slices of kidney showed no significant change in incorporation of tryptophan-1'-C^{14} into protein in the presence of ICSH. Also slices of testis showed no significant change in incorporation in the presence of ACTH (14 units per flask).

10. In the experiments reported in this section, the figures expressed as CPM per mg protein are the means of duplicate readings. In each case the TCA precipitable material was plated in duplicate and the difference between duplicates was less than 10 per cent.

11. In the experiments reported in 4, 5, 6, 8 and 9 above,

incubation of slices from each animal with acetate-1-C^{14} in separate flasks, was performed and revealed an increase in the incorporation of acetate-1-C^{14} into testosterone by slices exposed to the action of the gonadotrophic hormones in vivo or in vitro in each case. Table LIII shows this response in the case of experiments reported above in 6.

Table LIII

The Incorporation of Acetate-1-C^{14} into Testosterone by Slices of Testis from Rabbits Pretreated with ICSH.

Duration of Pretreatment	Testosterone CPM above unstimulated level per 100 mg protein
1 hour	4,682
2 hours	4,810
3 days	3,937

Conclusions

These findings support the following conclusions:

1. Puromycin in concentrations of 50 µg per ml and chloramphemicol in concentrations of 100 µg per ml, completely inhibit the response of slices of testis to ICSH *in vitro*.

2. Puromycin and chloramphemicol in concentrations of 100 ug per ml *in vitro* does not impair the response of slices of rabbit testis to previous stimulation by HCG *in vivo*.

3. Puromycin *in vivo* (100 mg per kg body weight) did not abolish the response of testis to HCG given as a single intravenous injection.

4. HCG administered *in vivo* for periods of 1 hour to 3 days caused a significant increase in the rate of incorporation of tryptophan-1'-C^{14} into protein by slices of rabbit testis *in vitro*.

5. ICSH administered *in vivo* for periods of 1 hour to 3 days caused a significant increase in the rate of incorporation of tryptophan-1'-C^{14} into protein by slices of rabbit testis *in vitro*.

6. ICSH *in vitro* caused a significant increase in the rate of incorporation of valine-1-C^{14} into protein by slices of rabbit testis in doses of 10^{-1} to 10 µg per flask. A less striking effect was seen in the case of tryptophan-1'-C^{14}.

7. That incorporation of amino acids into protein was being measured in these experiments was supported by the decrease in specific activity of TCA precipitable material seen when puromycin was added *in vitro*.

8. The specificity of these responses to HCG and ICSH was indicated by failure of slices of testis to respond to ACTH and by failure

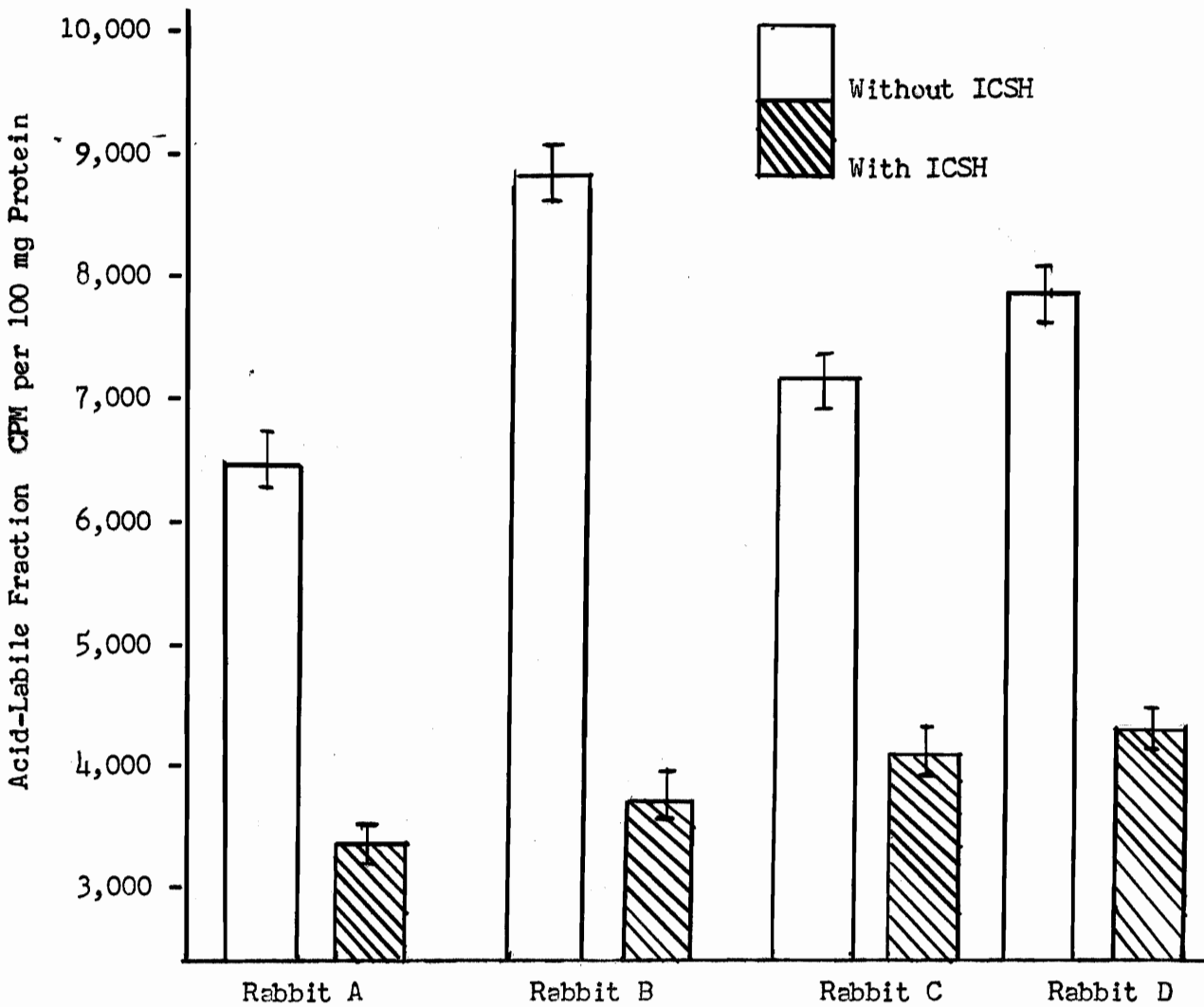
of slices of kidney to respond to ICSH.

9. A single intravenous injection of ICSH (1 mg) caused increase in the rate of incorporation of acetate-1-C^{14} into testosterone by slices of testis prepared one hour after injection.

Section 11

The Influence of ICSH upon the Rate of Incorporation of Acetate into Terpene Alcohols by Slices of Rabbit Testis *in vitro*.

Figure 11 shows the results of experiments in which the rate of incorporation of sodium acetate-1-C^{14} into terpene alcohols *in vitro* was studied with and without ICSH. The terpene alcohols were extracted as the so-called "acid-labile fraction" (page 109) following saponification and ether extraction.



(For caption see page 183)

Figure 11. The incorporation of acetate-1-C^{14} into terpene alcohols by slices of rabbit testis. Incubation was performed under standard conditions (page 99) and ICSH (10 µg per flask) was added to appropriate flasks. In each case a bar represents the mean of 4 flasks, the range being indicated by I. In each rabbit the usual isolation of radioactive testosterone was performed and slices were shown to have incorporated more acetate-1-C^{14} into testosterone in the presence of ICSH than in its absence.

Conclusion

These findings indicate that ICSH causes a decrease in the amount of acetate-1-C^{14} incorporated into terpene alcohols by slices of rabbit testis *in vitro*.

Chapter IV

DISCUSSION

It will be assumed from the first section of the experimental findings, that an effect <u>in vitro</u> of ICSH upon slices of rabbit testis has been established and consistently repeated. It will also be assumed that this effect is, at least in part, expressed as an increase in the rate at which acetate-1-C^{14} is incorporated into testosterone. Moreover, this decisive stimulation by ICSH indicates that directly or indirectly, the trophic hormone is capable of increasing the rate of steroid biosynthesis over the whole pathway from acetate to testosterone.

Furthermore, it will be assumed that the biosynthesis of testosterone in the testis is confined to the Leydig cells; the evidence for this assumption has been reviewed in detail (1,2). However, it is not possible at present to exclude the possibility that small amounts of androgen are secreted within the seminiferous tubules. Nevertheless, this possibility, for which there is no direct evidence, will be ignored in the present chapter. Since the synthesis of testosterone is largely confined to the Leydig cells, it is necessary to interpret the experiments of Chapter III accordingly, for these experiments have measured the activity of a few cells within a mass of inactive tissue. Various attempts have

been made to estimate the relative volume of the testis occupied by Leydig cells. Bascom and Osterud, in a careful study of several species, reported that the total interstitial tissue (blood vessels, fibrous tissue and Leydig cells) occupies between 3 and 10 per cent of the volume of the testis in the guinea pig (3). Slotopolsky and Schinz found Leydig cells to occupy 12 per cent of the volume of the human testis (4). The relative weight of Leydig cells is not accurately known.

It will further be assumed that the effect described is peculiar to gonadotrophic hormones, since other pituitary hormones are without effect in comparable concentrations. No final opinion can be expressed as to whether the capacity to stimulate incorporation of acetate into testosterone is confined to ICSH or is shared (unequally) by both gonadotrophic hormones. Since there can be no proof that either of the hormones used existed in a state of "absolute" purity and since bioassay can exclude only one part of ICSH in 6,000 parts of FSH (see page 95) and since a response has been obtained to as little as 10^{-4} µg of ICSH, it seems more logical to conclude that the preparations of FSH used were not entirely free of ICSH. Since the lowest dose of FSH to which a response was obtained was 10^{-3} µg (see page 132), it remains possible that contamination of FSH with traces of ICSH not detectable by bioassay, may account for the response observed to FSH. It will be considered that such contamination exists until proof to the contrary is obtained.

The experiments reported in Chapter III (Section 2), in which incorporation of acetate-1-C^{14} into testosterone was not found following the procedures of freezing at zero time or heating at 40°C for 30

minutes, suggest that the response measured in other experiments requires the activity of living cells.

If the capacity to stimulate the rate of incorporation of acetate into testosterone is a property peculiar to ICSH, it remains to be decided whether FSH can act as a synergist by allowing lower concentrations of ICSH to cause such stimulation than would otherwise be possible. It is clearly impossible to exclude synergism by means of the available data, since in the living animal the testis is subjected to the action of an unknown and perhaps variable concentration and mixture of gonadotrophins and the nature of this mixture may affect the behavior of slices incubated in vitro. In experiments in which the two hormones were added together, no evidence of synergism was revealed. However, in Experiment 57, preincubation with a subthreshold concentration of either hormone, followed by addition of a subthreshold concentration of the other hormone, apparently produced a synergistic effect. Again, however, until the possibility of contamination can be excluded, synergism must be regarded as an unconfirmed possibility.

While the existence of an action of ICSH in vitro can be regarded as established, the measurement of this response presents serious difficulties. The experimental data clearly indicate that the concentration of ICSH added in vitro does not bear a linear relationship to the response (Section 2*). The causes of this

* The sources of data mentioned in this chapter will be indicated by the section of Chapter III from which they are taken and will appear in the text in parentheses without the chapter number.

irregularity in the response are of two types, namely chemical and biological. Chemical causes include variable recovery of testosterone (56 to 71 per cent) and the impurity of the isolated testosterone revealed by recrystallization and gas chromatography.

Biological causes for the irregularity of the observed response are indicated by the variable rate of incorporation of acetate-1-C^{14} into testosterone by unstimulated slices and by variation in the response of slices from different animals. These variations impose two limitations upon the design of the present experiments and upon their interpretation. Firstly, only gross differences in the response of slices can be regarded as significant and secondly slices from one rabbit can be compared only with slices from the same animal.

In connection with the variable response to ICSH by slices, the observations made earlier concerning the small contribution of the Leydig cells to the total mass of the testis, are relevant. It is likely that the amount of Leydig cell tissue in a given weight of testis, varies from slice to slice.

In the present experiments sheep ICSH and human chorionic gonadotrophin have been used to stimulate rabbit testes. Recent studies by Knobil and Josimovich indicate that ICSH from species other than primates (sheep and horse) are active in the hypophysectomized male rhesus monkey and that human ICSH is active in the same species of monkey (5). The authors present evidence for certain quantitative differences between the efficacy of the human and the sub-primate preparations. Since the experiments reported in Chapter III were all performed with sheep ICSH and rabbit testis, such quantitative variation has been excluded between one experiment and

another. Moreover, these experiments indicate that the lack of species specificity reported by Knobil and Josimovich is also seen among sub-primate species.

The first clue which the present data offer as regards mechanism of action of ICSH, is the observation that the hormone can produce the same response from slices of testis *in vitro*, whether it is administered *in vivo* or added *in vitro*. In other words, some change can be produced *in vivo*, within one hour of an intravenous injection, which produces an effect *in vitro* without further stimulation. Moreover, when HCG is administered subcutaneously, this change persists for at least two days following the last injection (Section 3).

These observations lead naturally to the question of what the Leydig cell needs in order to incorporate acetate into testosterone. The answer may be given as follows:

1. Acetate.
2. The appropriate enzymes in active form.
3. The necessary cofactors in the required form.
4. A geometrical arrangement of these substances within the cell, such that the appropriate factors are present at the required time and in the appropriate part of the cell.

It is helpful to consider how a trophic hormone might influence these requirements.

1. It could enhance the availability of acetate to the cell.
2. It could make cofactors available to the cell.
3. It could affect the state of available cofactors.
4. It could make one or more enzymes more freely available by synthesis or activation.

5. It might affect the geometrical disposition of the required factors within the cell.

6. The hormone could exert more than one effect.

If ICSH acted by making acetate available to the cell, the hormone would presumably cause a specific stimulation of transport of this substrate across the cell membrane. However, ICSH administered *in vivo*, can stimulate the incorporation of acetate-1-C^{14} into testosterone *in vitro* 24 hours after the last injection or one hour after a single intravenous injection (Section 10). It might be argued that intense stimulation *in vivo* would cause the cell to become flooded with unlabeled acetate and increase in the uptake of acetate-1-C^{14} *in vitro* would not be expected. Moreover ICSH does not stimulate the rate of incorporation of acetate-1-C^{14} into fatty acids (Section 6), so that there is no evidence of increased disposal of absorbed acetate into pathways other than steroid synthesis (although other possible routes of disposal were not tested). However, it is possible to conceive that ICSH enhances the permeability of Leydig cells to acetate without causing saturation at any one time. How much of the acetate used in steroid biosynthesis *in vivo* enters the cell from without and how much is derived from endogenous metabolism, is unknown and a possible influence of ICSH upon the intracellular distribution of acetate cannot be excluded.

Since stimulation of the rate of incorporation of acetate into testosterone can be demonstrated in a medium consisting of Krebs-Ringer bicarbonate buffer and acetate, the only other factors susceptible to enhanced permeability are inorganic ions. It is true that divalent metal ions are required for the activity of enzymes concerned

in steroid biosynthesis (25) but there is no evidence that intracellular concentrations of such ions are rate-limiting in the synthesis of testosterone.

In Section 9 of Chapter III it was seen that under the conditions tested, ICSH showed no consistent effect upon the equilibration of D-xylose-1-C^{14} or α-aminoisobutyric acid-1-C^{14} across the cell membranes of testicular slices. Since these substances are not significantly metabolized by mammalian tissues, they have been used as model compounds for the study of membrane transport (6,9). D-xylose would be expected to reflect the behaviour of sugars and has been used for this purpose in the study of the action of insulin (6). On the other hand, α-aminoisobutyric acid has been similarly employed as a model for the behaviour of amino acids (9).

In keeping with the failure of ICSH to enhance the transport of D-xylose into testicular cells, is the finding that glucose does not enhance the response of slices of testis to ICSH (Section 7), so that the hormone is not likely to act by promoting the absorption of sugars from the incubation medium. On the other hand, glucose has been shown to enhance the response of the adrenal cortex to ACTH (11) and it has not been possible to show that ACTH affects the transport of D-xylose across the membranes of adrenal cells in vitro (8). Hechter and coworkers were able to show that the penetration of D-xylose into the adrenal cells of hypophysectomized rats was enhanced by injection of ACTH in vivo (7). However, the same group was unable to demonstrate an effect in vitro using slices which showed increased corticosteroid production in the presence of ACTH (8). It is, therefore, likely that the effect demonstrated in vivo is not specific,

and that the response to ACTH in vitro (increased steroid production), cannot be attributed to enhanced transport of glucose, although this possibility cannot be entirely excluded since D-xylose may not reflect the behavior of glucose in the case of the response of the adrenal to ACTH.

In the case of the adrenal gland it has also been shown that freezing prevents slices from responding to ACTH but enhances their response to TPNH (10) and that calcium ions also increase the response to ACTH (10). Although it might be supposed that freezing alters the permeability of the adrenal cells, it has been shown that freezing also stimulates adrenal homogenates (10). By contrast, freezing of slices of testis does not affect the response to ICSH nor does freezing enable reduced TPN to stimulate the rate of incorporation of acetate-1-C^{14} into testosterone, ~~nor does~~ freezing by itself, does not stimulate incorporation of acetate-1-C^{14} into testosterone (Section 7).

While the testis differs from the adrenal in response to the stimuli mentioned, homogenates of both organs show an increase in steroid production in the presence of reduced TPN (Section 8). In this respect the testis differs from beef corpus luteum, slices of which respond to TPN and to TPNH (12). Slices of testis failed to respond to oxidized or to reduced TPN in concentrations of from 10^{-3}M to 5×10^{-3}M (Section 7). Similarly, slices of testis do not respond to DPN or to DPNH, each being added in concentrations of from 10^{-3}M to 5×10^{-3}M. Three explanations for the failure of slices of testis to respond to reduced TPN can be offered, namely:

(i) Leydig cells differ from luteal cells in not being permeable to TPNH.

(ii) The germinal epithelium of the testis, being present in much greater quantity than the Leydig cells, is capable of removing much of the available TPNH present in the medium.

(iii) In the whole cell, the total concentration of TPN or the proportion present in the reduced form, are not rate-limiting in steroid biosynthesis.

On the other hand, reduced TPN stimulates the conversion of cholesterol-4-C^{14} to testosterone by a homogenate of testis (Section 8). If the intracellular concentration of TPN or of the proportion present in the reduced form is rate-limiting for steroid biosynthesis, one would expect reduced TPN to stimulate the conversion of acetate-1-C^{14}, mevalonate-2-C^{14} and butyrate-1-C^{14} to testosterone by a homogenate. Yet, in the presence of 1.5×10^{-2}M reduced TPN, no incorporation of these substrates into testosterone was detected. This concentration is ten times as great as that used by Bucher et al. to bring about the biosynthesis of cholesterol from acetate by homogenate of liver (13). This objection can be met by arguments based upon the existence of intracellular compartments. It may be supposed that in the testis, TPNH is rate-limiting for one or more steps before cholesterol and that these steps are isolated in compartments which are not destroyed by the process of homogenization used in these experiments and which are denied free access to the general pool of reduced TPN within the homogenate. Alternatively such compartments may contain sufficient TPN but following homogenization, may be beyond the reach of mechanisms required for its reduction.

Again, it can be argued that some intermediate between acetate and cholesterol may be formed, but may become "lost" in the homogenate

and unable to find the appropriate enzyme for which it acts as substrate. In the whole cell, such an intermediate will be formed at a site which places it close to the required enzyme; this arrangement will be lost in a subcellular preparation. Such a concept gains force from the fact that the contents of the Leydig cells will be greatly diluted by the contents of the germinal cells.

The stimulating influence of reduced TPN upon the rate of conversion of cholesterol-4-C^{14} to testosterone by a homogenate (Section 8) cannot necessarily be taken to indicate that the availability of TPNH in the whole cell is rate-limiting for the steps between cholesterol and testosterone. A major factor in the rate of a chemical reaction is the frequency of collision between reacting molecules. The cell uses at least two devices to enable reactions to occur rapidly, in spite of low concentrations (hence infrequent collisions) of reactants. Firstly, enzymes stimulate biological reactions and secondly, it is likely that subcellular architecture provides a particular arrangement of reactants which proves most favorable for frequent encounter between enzyme and substrates. The disruption of cellular architecture caused by homogenization may cause concentrations of TPNH which are greater than rate-limiting when arranged in a specific way, to become rate-limiting when their arrangement is random. Hence, the homogenate provides something between the random encounters of a test tube reaction on the one hand and the organized "factory belt" of the whole cell on the other, since disorganization is not necessarily complete.

It is not at present possible to account for these differences between the behavior of adrenal, ovarian and testicular tissue.

However, there is reason to believe that the biosynthesis of steroids may differ in adrenal and testicular tissue. Kahnt and coworkers for example, demonstrated that adrenal tissue makes equal use of two alternate pathways from pregnenolone to C_{19} steroids (14). On the other hand, the data of Eik-Nes, from perfusion studies in the dog, indicate that when progesterone-4-C^{14} and 17-hydroxypregnenolone-7-H^3 are used together as substrates for the testis, the resulting testosterone shows approximately five times as much H^3 as C^{14} (15). This difference in alternate pathways may affect the response of the two tissue to various stimulating agents.

Again, luteal tissue consists of large cells set in a vascular bed of ovarian tissue and these cells have been intensely stimulated by ICSH in vivo; such cells may differ from Leydig cells in permeability to reduced TPN. Finally, species differences between the rat, the cow and the rabbit may be relevant.

The role of reduced TPN in the rate of steroid biosynthesis is still a subject for dispute. In the adrenal, Glock and McClean found that a high proportion of the TPN present was in the reduced form (16), although the validity of the method used by these authors, for estimating the relative proportions of reduced and oxidized forms has been questioned (17) and the ubiquitous nature of stress as a stimulus in laboratory animals makes it difficult to exclude stimulation in vivo by endogenous ACTH.

It is the contention of Haynes that ACTH increases the proportion of the TPN of the adrenal in the reduced form (18). It is not possible to establish this contention by adding excess TPNH to adrenal homogenate, nor by adding cyclic AMP to slices. Among the stimuli

which increase the production of cortocosteroids by the adrenal cortex are freezing, calcium ions, proteolysis and glucose (10,11). It cannot be said that this response is to be regarded as highly specific.

The failure of cyclic AMP to increase the rate of incorporation of acetate-1-C^{14} into testosterone (Section 7) by slices of rabbit testis and cholesterol-4-C^{14} into testosterone by a homogenate (Section 8) may mean no more than that Leydig cells are not permeable to cyclic AMP and that cyclic AMP cannot act in a subcellular system (as proved to be the case with the adrenal (19)). Furthermore, cyclic AMP may not stimulate the activation of testicular phosphorylase.

It was observed in Section 6 of Chapter III that ICSH could stimulate the rate of incorporation of acetate-1-C^{14} into testosterone without significantly changing the rate of incorporation of acetate-1-C^{14} into an uncharacterized fatty acid fraction. If TPN is present in the Leydig cell as a free pool available to all metabolic activities which require this cofactor, it would be expected that any change which increased the available TPNH in this pool, would stimulate any reaction in which the availability of TPNH is rate-limiting. If ICSH directly increases the TPNH present in a general intracellular pool, it would be expected to promote such reactions, but if ICSH stimulates steroid synthesis by some other mechanism it could inhibit other reactions using TPNH by causing steroid synthesis to compete successfully for available TPNH. The failure of ICSH significantly to alter the rate of incorporation of acetate-1-C^{14} into fatty acids can, therefore, be explained in three ways, namely:

1. ICSH does not specifically enhance the availability of reduced TPN.

2. Fatty acid synthesis is geometrically isolated from steroid synthesis so that the two activities do not compete for a common supply of reduced TPN.

3. The availablity of reduced TPN is not rate-limiting for fatty acid synthesis.

There is little to support the idea that ICSH acts by activating an enzyme present in the testis in an inactive form. In the case of the adrenal cortex the stimulating influence of calcium ions, freezing and proteolysis have been suggested as possible non-specific activators of adrenal enzymes (10). There is no evidence that ICSH possesses proteolytic properties; a successful search for proteolytic enzymes in the adenohypophysis has been made (20) and might perhaps have revealed proteolytic properties of ICSH if these existed. Neither is any evidence available to suggest that ICSH activates an enzyme by making a divalent metal ion available or by causing such a drastic change in molecular structure as, for example, dimerization of phosphorylase.

The first evidence that the action of ICSH may result from protein synthesis came from the observation that puromycin and chloramphemicol were capable of inhibiting the response of slices of testis to ICSH but were without effect upon the stimulation of slices seen following administration of HCG <u>in vivo</u> (Section 10). The last observation shows that HCG <u>in vivo</u> causes a change within the Leydig cells which puromycin and chloramphemicol cannot subsequently inhibit <u>in vitro</u> and since both substances are selective inhibitors of protein

synthesis, it is logical to conclude that they inhibit the synthesis of protein which is necessary for the activity of ICSH, but that they cannot inhibit the action of the newly synthesized protein nor do they inhibit the incorporation of acetate into testosterone by any other mechanism. Unfortunately the limited and variable incorporation of acetate into testosterone shown by unstimulated slices does not permit the direct experimental approach of adding puromycin to unstimulated slices in order to demonstrate that the antibiotic does not inhibit steroid synthesis by such slices. Moreover, puromycin does not inhibit the rate of conversion of cholesterol-4-C^{14} to testosterone by a homogenate of testicular tissue and such a preparation is not stimulated by addition of ICSH (Section 8).

More is known about the detailed mechanism of action of chloramphemicol (21) than about that of puromycin and most of the early studies were performed with microorganisms since both compounds are widely used as antibiotics. Chloramphemicol produces complete inhibition of the growth of bacteria in concentrations of 1-10 µg per ml, in contrast to the wide variation in the dose of penicillin required to produce the same effect (21). All species of bacteria have been shown to be sensitive to chloramphemicol, although resistant strains are well known. The concentration required to inhibit protein synthesis in plants and animals is greater and more variable (21).

A recent review reports a vast number of experiments which established chloramphemicol as a specific inhibitor of protein synthesis (21). The argument is based upon exclusion of other possible mechanisms of inhibition of growth. For example, chloramphemicol does not inhibit the oxidation of numerous substrates tested, nor the

synthesis of glucose, ATP, amino acids and numerous other important cellular metabolites. The synthesis of purines, pyrimidines, nucleotides and nucleic acids is likewise unimpaired by chloramphemicol. The accumulation of free amino acids within the cell is normal in the presence of chloramphemicol and the "free amino acid pool" increases at a rate approximately equal to the rate at which amino acids are incorporated into protein in control incubations without chloramphemicol.

On the other hand, 10 µg of chloramphemicol per ml of medium causes a 95-100 per cent inhibition of protein synthesis by growing cells. An impressive list of enzymes has been studied and in more than 20 of these it has been possible to show that enzyme activity is maintained but synthesis is impaired by chloramphemicol. In some cases, it has been shown that the activation of inactive enzyme precursors proceeds normally in the presence of the antibiotic. Finally, in cells which are not growing, it has been shown that chloramphemicol does not affect the degradation of protein (21).

Of the three well recognized steps in protein synthesis (amino acid activation, transfer to soluble RNA and transfer to protein), it is likely that chloramphemicol inhibits the last of these, although at what point and in what manner remain uncertain. The RNA synthesized in the presence of chloramphemicol is unstable and the properties of the ribosomes are altered (21).

It has also been shown that puromycin is a specific inhibitor of protein synthesis and it is believed to act at the same step in the synthesis of protein as chloramphemicol (23,24). Nathans and Lipmann found that 200 µg/ml of puromycin caused more than 90 per cent inhibition of the incorporation of labeled amino acids into TCA precipitable material by liver microsomes (24). Hot TCA has

been found necessary to prevent precipitation of amino acids bound by non-specific forces (21,22).

The finding that chloramphemicol and puromycin inhibit the response of slices of testis to ICSH makes it likely that this response involves protein synthesis. More direct evidence for this idea is shown in the experiments reported in Section 10 of Chapter III in which ICSH was shown to stimulate the rate of incorporation of labeled amino acids into material precipitated by hot TCA. This stimulation was seen after injection of ICSH and HCG *in vivo* for 3 days and in both cases after a single intravenous injection. In these experiments one testis from each animal served as the control for the response of the second testis to the trophic hormone. Since it was possible to demonstrate increased incorporation of amino acids into protein within one hour of a single intravenous injection, such synthesis of protein could be involved in the increased rate of incorporation of acetate-1-C^{14} into testosterone, which follows a single intravenous injection of ICSH (Section 10).

Studies of the incorporation of amino acids into protein by slices of rabbit testis with and without ICSH *in vitro* showed that a significant increase in the rate of such incorporation occurred within 20 minutes, in the presence of ICSH. In these experiments valine showed a greater response than tryptophan. Since the increase in the rate of incorporation of acetate-1-C^{14} into testosterone produced by ICSH *in vitro* requires at least one hour of incubation in the present system (Section 2), the increased incorporation of amino acids into protein is sufficiently rapid to be involved in this response.

The decisive inhibition of the incorporation of amino acids into protein in the presence of puromycin, in both stimulated and unstimulated slices, indicates that true incorporation was being measured. Further reassurance was provided by comparing stimulated and unstimulated slices from one animal. The importance of such reassurance is indicated by the possibility of artifictitious incorporation, as described by Simkin (26).

If protein synthesis is an integral part of the response of Leydig cells to ICSH, it might be conjectured that the newly synthesized protein is an enzyme involved, directly or indirectly, in steroid biosynthesis. This raises the question of which part of the biosynthetic pathway is subjected to stimulation by ICSH. It has not been possible to demonstrate stimulation by ICSH of the conversion of cholesterol-4-C^{14} to testosterone by a homogenate and the conversion of cholesterol-4-C^{14} to testosterone by an unstimulated homogenate cannot be attributed to the presence of whole cells, since cholesterol-4-C^{14} is not converted to testosterone by slices of rabbit testis (presumably because it fails to enter the cells (Section 2)). On the other hand, injection of HCG *in vivo* for periods of from one hour to 3 days, causes increase in the rate of incorporation of acetate-1-C^{14} into testosterone by slices *in vitro* (Section 10). However, injection of HCG *in vivo* does not cause incorporation of acetate-1-C^{14} or of mevalonate-2-C^{14} into testosterone by a homogenate, either whole homogenate or the supernatant layer left after centrifugation at 650 x g for 15 minutes (Section 8). When pregnenolone-7-H^3 was used as substrate for a homogenate it was not possible to show that ICSH *in vitro*, increased the rate of conversion of this substrate to testosterone (Section 8).

Finally, no significant radioactivity was detected in the "cholesterol fraction", removed by column chromatography, when acetate-1-C^{14} was used as substrate for a homogenate but significant radioactivity was found in this fraction when acetate-1-C^{14} was used as substrate for slices (Section 8).

Perhaps the simplest explanation for these observations is that ICSH acts at some stage before the synthesis of cholesterol and hence injection of HCG *in vivo* does not stimulate the conversion of cholesterol-4-C^{14} to testosterone by a homogenate *in vitro*. Slices of testis, in the presence of ICSH, show increased incorporation of acetate-1-C^{14} into testosterone, since the trophic hormone acts before cholesterol and once an enzyme, normally present in rate-limiting amounts, is synthesized, the conversion of acetate to testosterone is stimulated in the whole cell. In the homogenate, however, certain early intermediates (before cholesterol), become "lost" by dilution and stimulation is not seen. Presumably the cell architecture is not so far destroyed as to prevent cholesterol-4-C^{14} undergoing the changes required to produce testosterone.

Alternatively, it is possible that stimulation by ICSH is not seen in the homogenate because this is prepared by the method of Bucher *et al.* for sterol synthesis in liver (13) and makes inadequate provision of cofactors required for protein synthesis.

The observation that the acid-labile fraction (page 109) shows less radioactivity during the stimulation of steroid biosynthesis by ICSH (Section 11) would suggest that ICSH acts after the formation of farnesyl pyrophosphate. This fraction measures dimethylallyl alcohol, isopentenyl alcohol, geranol and farnesol (27).

During the experiments reported, the action of ICSH *in vitro* on slices of testis was used to provide certain information not relevant to the mechanism of action of the hormone.

1. Testicular tissue in the rabbit contains the fatty acids, palmitate, palmitoleate, stearate and oleate and adrenal tissue contains palmitate, stearate and oleate in the same species. The relative proportions and total quantities of these acids are not significantly altered in either tissue during increased steroid biosynthesis *in vitro*.

2. Aqueous solutions of ICSH, whether cold (18°C) or frozen, lose gonadotrophic activity after a period of several weeks.

3. Three preparations of sheep ICSH, although prepared by different methods, showed no significant difference in potency in the present assay system when tested in two different concentrations.

4. Pituitary tissue stimulates the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*. Moreover, during a two hour period of incubation approximately half of the gonadotrophic activity present in slices of pituitary tissue is released from the cells into the medium and this fraction is significantly increased by the presence of hypothalamic tissue in the medium (Section 5). As yet, it has not been possible to compare different areas of hypothalamic tissue in detail but supraoptic nucleus, paraventricular nucleus and median eminence all appear to be active; vasopressin was without demonstrable effect (Section 5).

5. The puzzling observation was made that FSH *in vitro* inhibits the stimulation produced by HCG administered *in vivo*, upon the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis (Section 3). On the other hand, FSH *in vitro* does not

inhibit the conversion of cholesterol-4-C^{14} to testosterone by homogenate of testes from animals which had received HCG *in vivo* (Section 8).

These observations do not, at present, admit of ready explanation. The effects of HCG in the whole animal (page 26) are complex and probably include stimulation of the germinal epithelium. It may be that the germinal cells, having been stimulated *in vivo*, compete with Leydig cells for acetate when slices are exposed to FSH *in vitro*, since the latter may cause further stimulation of the germinal cells, *in vitro*. This effect of FSH is not inhibited by chloramphemicol and, therefore, probably does not involve protein synthesis. Again, FSH does not inhibit the homogenate of testes from animals pretreated with HCG, so that the hormone does not inhibit the later steps in the biosynthetic pathway in a subcellular system.

Versene and cysteine have been shown to decrease the potency of FSH (page 16) but the ommision of these substances from the medium in which a testicular homogenate was prepared did not affect the rate of conversion of cholesterol-4-C^{14} to testosterone by such an homogenate in the presence of ICSH (Section 8).

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SUMMARY

1. Interstitial cell-stimulating hormone increases the rate at which acetate-1-C^{14} is incorporated into testosterone by slices of rabbit testis *in vitro*.

2. Freezing does not increase the rate at which acetate-1-C^{14} is incorporated into testosterone by slices of rabbit testis *in vitro*.

3. Addition of pyridine nucleotides (oxidized or reduced) or of cyclic AMP, does not increase the rate of incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis *in vitro*.

4. Freezing, addition of glucose or reduced TPN to the medium and preincubation with or without glucose, neither impair nor enhance the response of slices of rabbit testis to ICSH *in vitro*.

5. The response of slices of rabbit testis to ICSH *in vitro*, does not involve increase in the rate of incorporation of acetate-1-C^{14} into fatty acids.

6. Homogenate of rabbit testis is capable of converting cholesterol-4-C^{14} and pregnenolone-7-H^3 to testosterone. The rate of conversion of these substrates to testosterone is not increased by ICSH but the conversion of cholesterol-4-C^{14} to testosterone is increased by addition of reduced TPN.

7. Administration of HCG or ICSH *in vivo* causes increase in the rate at which slices of rabbit testis incorporate acetate-1-C^{14} into testosterone *in vitro*.

8. Puromycin and chloramphemicol prevent the response of slices of rabbit testis to ICSH *in vitro*, but do not inhibit the increased rate of incorporation of acetate-1-C^{14} into testosterone by slices *in vitro*, following administration of HCG *in vivo*.

9. HCG *in vivo* and ICSH both *in vivo* and *in vitro*, increase the rate at which the amino acids valine-1-C^{14} and tryptophan-1'-C^{14} are incorporated into protein by slices of rabbit testis *in vitro*.

10. The combined radioactivity of the terpene alcohols (isopentenyl alcohol, dimethylallyl alcohol, geranol and farnesol) is lower in slices of rabbit testis stimulated by ICSH *in vitro* than in unstimulated slices, with acetate-1-C^{14} as substrate.

11. Slices of canine anterior pituitary tissue release gonadotrophic hormone into the medium during incubation in Krebs-Ringer bicarbonate buffer *in vitro*. The rate of release of gonadotrophic hormone from such slices is increased by addition of slices of hypothalamic tissue to the medium.

In conclusion, present evidence may be said to indicate that ICSH acts upon testicular tissue by stimulating protein synthesis and that the effect of this stimulation is active at some stage in the biosynthetic pathway after farnesyl pyrophosphate.

RESEARCH PROPOSALS

I. Digestion of human chorionic gonadotrophin (HCG) with pepsin has been reported to yield dialysable fragments which possess biological activity[1]. So far, these investigations have not been reported in detail but fragments resulting from the controlled digestion of HCG by pepsin, could be tested *in vitro* with slices of rabbit testis to find which fragments were capable of stimulating the rate of incorporation of acetate-1-C^{14} into testosterone. The method of assay to be used in these studies has been reported in the accompanying thesis.[2] The fragments of lowest molecular weight which are active *in vitro* would be suitable for studies of amino acid sequence.

[1]Wettstein, A. and Benz, F. U. S. Pat. #2734017, 1956.

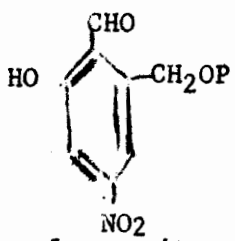
[2]Hall, P. F. On the Mechanism of Action of Interstitial Cell-Stimulating Hormone. PhD. Thesis, University of Utah, 1961.

II. Four of the eight phosphate groups found in phosphorylase a are present in the form of pyridoxal phosphate. Fischer et al. have shown that pyridoxal phosphate is not found in phosphorylase as a Schiff base but as a secondary amine derivative[1]. Pyridoxal phosphate can be removed from the enzyme with complete loss of enzymatic activity, and can be reintroduced into the molecule with restoration of activity[2]. These findings indicate that the mechanism of action of pyridoxal phosphate in phosphorylase differs from the conventional mechanism proposed to explain its many activities in other biological systems[3]. Since pyridoxal phosphate can be reversibly removed from phosphorylase, it is proposed to replace this cofactor by a number of model compounds to see what structural properties are required to restore enzymatic activity.

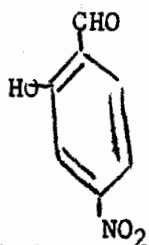
Fortunately numerous model compounds have been prepared to study other activities of pyridoxal[3]. The following are of interest:

(I) Replacement of pyridine nitrogen by a nitro group:

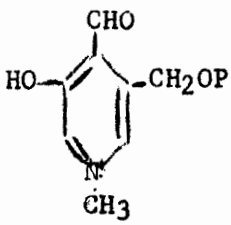
(a)



(b)

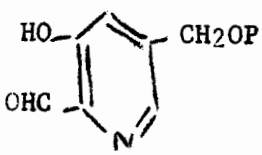


(II) Fixation of one nitrogen valence by a methyl group:

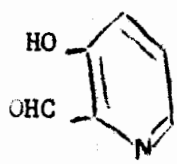


(III) Change in the spatial relationship between pyridine nitrogen and the aldehyde group:

(a)

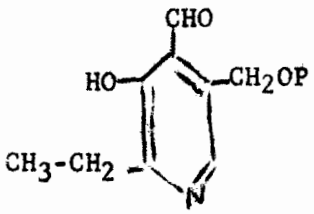


(b)

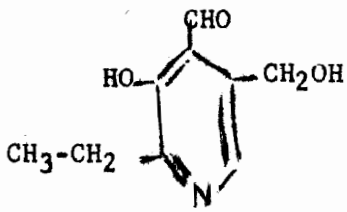


(IV) Change in methyl side chain:

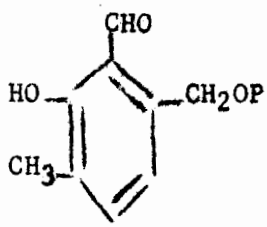
(a)



(b)



(V) Removal of Nitrogen:



[1]Kent, A. B., Krebs, E. G. and Fischer, E. H. J. Biol. Chem., 232: 549, 1958.

[2]Cori, C. F. and Illingworth, B. Proc. Nat. Acad. Sci., 43: 547, 1957.

[3]Snell, E. E. Vitamins and Hormones XVI: 77, 1958.

III. In experiments in which the influence of interstitial cell-stimulating hormone upon testicular tissue *in vitro* is studied, two major difficulties arise, namely the bulk of inactive tissue present in the form of seminiferous epithelium and the possible influence of variation in the circulating concentrations of endogenous gonadotrophins in the living animal. One approach which may reduce the interference arising from these factors is the use of hypophysectomized animals.

It would be necessary to study the synthesis of steroids by testicular tissue at varying intervals after hypophysectomy, since resistance has been described to the administration of ICSH *in vivo* if the time between operation and the commencement of treatment exceedes seven to ten days[1].

In this way it may be possible to find a time after operation at which the Leydig cells show maximal sensitivity to ICSH. At such a time, studies of the mechanism of action of ICSH could be conducted along the lines reported in the accompanying thesis[2], in the hope that the Leydig cells may be more sensitive to ICSH than in the intact animal. It is proposed to study especially the capacity of slices of testis from hypophysectomized animals to incorporate labeled amino acids into protein and the response of a homogenate of testis from such animals to reduced TPN. In this way it may be possible to uncover the first response to ICSH.

[1]Simpson, M. E., Li, C. H. and Evans, H. M. Endocrinology, 30: 969, 1942.

[2]Hall, P. F. On the Mechanism of Action of Interstitial Cell-Stimulating Hormone. PhD. Thesis, University of Utah, 1961.

IV. Although thyroxine has been shown to influence the activity of many enzymes, two of these are of special interest. TPN-cytochrome reductase activity is significantly reduced by thyroidectomy and this reduction is made good by feeding thyroxine[1]. The enzyme α-glycerophosphate dehydrogenase is increased in liver 4 to 7 times following feeding with thyroxine. Ethionine inhibits this response, suggesting that protein synthesis is involved[2].

Recent studies of amphibian metamorphosis have shown that thyroxine also stimulates the synthesis of carbamyl phosphate synthetase and that thiouracil inhibits this synthesis by becoming incorporated into RNA[3].

Since the mechanism of action of thyroxine is a subject of considerable interest and since no biochemical factor common to its role in metamorphosis and its role in the adult animal has been found, it will be of interest to see whether thiouracil can impair the response of TPN-cytochrome reductase to thyroxine in the thyroidectomized animal and the response of α-glycerophosphate dehydrogenase to thyroxine feeding.

[1]Phillips, A. H. and Langdon, R. G. Biochim. Biophys. Acta, 19: 380, 1956.

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[3]Park, W. K., and Cohen, P. P. J. Biol. Chem., 236: 531, 1961.

V. Increased biosynthesis of steroids requires an increased supply of reduced TPN and although this could be provided in a number of ways, present interest centers around mechanisms which could stimulate the rate of disposal of glucose-6-phosphate by way of the pentose phosphate pathway. It is known that ACTH depletes the adrenal cortex of glycogen but it has not been possible to show whether the resulting increase in liberation of glucose units is a primary effect of ACTH which stimulates steroid production or a secondary effect resulting from a feedback mechanism by which it is coupled to the steps of steroid biosynthesis in such a way as to maintain TPN in a state of reduction.

Cohen has shown that the oxidative disposal of glucose by resting Escherichia coli cells is predominantly by the Embden-Meyerhof pathway, while rapidly growing cells chiefly use the pentose phosphate pathway[1]. Allen and Powelson have shown that in bacteria, growth, as distinct from cell division, is associated with a similar shift in favor of the pentose phosphate pathway[2].

It is proposed to employ the methods described by these authors to measure the relative disposal of glucose by the two pathways mentioned in the adrenal cortex <u>in vitro</u>, with and without ACTH. If the proportion of glucose units entering the two pathways is unchanged by ACTH, the possibility of a feedback mechanism must be considered. If ACTH increases the proportion of glucose entering the pentose phosphate pathway, a primary effect of the hormone on carbohydrate metabolism is likely.

[1]Cohen, P. S. Phosphorus Metabolism. Vol. I., page 148. Ed. McElroy and Glass. Johns Hopkins Press, Baltimore, 1951.
[2]Allen, S.H.G.,Jr. and Powelson, D. J. Bacteriology, 75: 184, 1958.

VI. When male rats are fed a diet containing 2% cholesterol, considerable depletion of the liver depot of vitamin A is observed. This phenomenon is more clearly seen in immature male rats and is not seen in females, immature or adult[1]. It would seem that three explanations can be suggested to account for these observations:

1. Cholesterol impairs the intestinal absorption of vitamin A.
2. The storage of cholesterol requires the metabolic disposal of vitamin A.
3. Cholesterol successfully competes with vitamin A for some aspect of hepatic storage. Both substances are transferred to the Kupfer cells from the reticuloendothelial cells of the liver.

Since the accumulation of cholesterol in the body is one of the important fields of current medical research, the striking findings mentioned above suggest a series of experiments to explore the mechanisms involved in the storage of cholesterol and vitamin A.

In the first place it is proposed to implant pellets containing vitamin A in the spleens of immature rats during a period of cholesterol feeding in order to exclude the first possibility mentioned above. If it turns out that diminished intestinal absorption of vitamin A is not responsible for the phenomenon mentioned, perfusion of whole rat liver would be suggested as the next experimental procedure. The rate of uptake of vitamin A from the perfusing fluid would be measured in livers from animals fed a control diet and from those receiving extra cholesterol. Again, cholesterol in various concentrations could be added to the perfusing fluid together with various doses of vitamin A.

It has usually proved easier to study the action of vitamin A in depleted animals[2]. Vitamin A pellets could be implanted in the spleens of depleted rats and the influence of cholesterol feeding could be studied to determine whether cholesterol could affect the deposition of vitamin A in such rats. Since vitamin A-2-C^{14} is available, the experiment could be conducted over a wide range of dosage.

These studies are seen as preliminary to detailed exploration of chemical factors involved in the storage of cholesterol and vitamin A. The subsequent experiments would be planned on the basis of the findings from these preliminary investigations.

[1]Green, B., Horner, A. A., Lowe, J. S. and Morton, R. A. Biochem. J., 67:234, 1957.

[2]Moore, T. Vitamin A. Elsevier Publishing Co., Amsterdam, 1957.